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Association of the FcγRIIB-nt645+25A/G polymorphism with the expression level of the *Fc*γRIIb receptor, the antibody response to *Porphyromonas gingivalis* and the severity of periodontitis

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Background and Objective: Human FcγRIIb is an immunoglobulin G (IgG) receptor that inhibits the activation of B lymphocytes through cross-linking with the B-cell receptor via immune complexes. This function acts as a negative regulator of antibody production. Our previous studies have demonstrated the gene polymorphisms in FcγRIIb to be associated with periodontitis. In this study, we presented a polymorphism – *FcγRIIB*-nt645+25A/G (rs2125685) – in intron 4 and analyzed its functional relevance to periodontitis. We examined whether the *FcγRIIB*-nt645+25A/G polymorphism is associated with periodontal parameters, the IgG response to the periodontopathic bacterium *Porphyromonas gingivalis* and/or the expression level of FcγRIIb on peripheral B lymphocytes.

Material and Methods: Thirty-two patients with chronic periodontitis were genotyped with nested PCR and by direct sequencing of genome DNA. The levels of serum IgG and of specific IgG subclasses for *P. gingivalis* sonicate and for the recombinant 40-kDa outer membrane protein (OMP) were determined. The expression levels of FcγRIIb on peripheral B lymphocytes from 19 healthy donors were measured by flow cytometry.

Results: Patients with the *FcγRIIB*-nt645+25AA genotype showed significantly higher mean clinical attachment levels compared to patients with

the Fcγ*RIIB*-nt645 + 25GG genotype ($p = 0.003$) and a significantly lower IgG response to *P. gingivalis* sonicate and to the 40-kDa OMP. The expression levels of FcγRIIb protein on the cell surface in peripheral B lymphocytes were higher in healthy donors with the *Fc*γ*RIIB*-nt645 + 25AA genotype than in those with the *Fc*γ*RIIB*-nt645 + 25GG genotype ($p = 0.03$).

Conclusion: The higher expression levels of FcγRIIb in subjects with the *Fc*γ*RIIB*-nt645 + 25AA genotype may induce a lower level of production of IgG against *P. gingivalis* and therefore more severe periodontitis.

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The receptors for the Fc region of immunoglobulin G (IgG) (FcγRs) are expressed by many immune cells (1). FcγRIIb is the only inhibitory Fc receptor that has an immunoreceptor tyrosine-based inhibitory motif on the cytoplasmic tail (2). FcγRIIb has been shown to act as a negative-feedback regulator of antibody production by inhibiting B-cell antigen receptor-elicited activation signals via the immunoreceptor tyrosine-based inhibitory motif through IgG immune complexes (3,4). Deficiency of FcγRIIb in mice was shown to be associated with severe inflammation triggered by immune complexes (5,6).

In a previous study we reported novel polymorphisms in the human *Fc*γ*RIIB* gene (7). Enrichment of 232T in the *Fc*γ*RIIB*-I232T (rs1050501) polymorphism was associated with susceptibility to periodontitis in Japanese subjects (8). The FcγRIIB-I232T polymorphism exists in exon 5, the transmembrane region, and has been reported to change affinity to lipid rafts (9,10). Among Japanese patients with chronic periodontitis, FcγRIIB-232T carriers had a significantly lower IgG2 response to the *Porphyromonas gingivalis* 40-kDa outer membrane protein (OMP) compared with noncarriers (11).

Recently, we investigated the prevalence of five polymorphisms in the *Fc*γ*RIIB* gene (-343G/C; nt645 + 7C/A; nt645 + 25A/G; nt646-184; and I232T) and the prevalence of *Fc*γ*RIIA*-131R/H, *Fc*γ*RIIA*-176F/V and *Fc*γ*RIIIB*-NA1/NA2 gene polymorphisms in pregnant Japanese women. We found that the *Fc*γ*RIIB*-nt645 + 25A/G (rs2125685) polymorphism, one of the *Fc*γ*RIIB* polymorphisms reported originally (7), showed significant associations with both preterm birth and periodontitis (12). The polymorphism *Fc*γ*RIIB*-nt645 + 25A/G exists in intron 4, between exon 4 and exon 5 (the number of nucleotides was indicated as nt645 + 26 in the previous study by Yasuda *et al.* for the same polymorphism described as *Fc*γ*RIIB*-nt645 + 25A/G in the present study). Although the *Fc*γ*RIIA*-131R/H polymorphism was also associated with periodontitis in pregnant women, the other *Fc*γ*R* polymorphisms showed no significant association with periodontitis or preterm birth (12). In the previous case–control study performed by Yasuda *et al.* (8), the *Fc*γ*RIIB*-nt645 + 25A/G polymorphism did not show a significant association with the prevalence of aggressive or chronic periodontitis. However, use of controls younger than patients may have resulted in the effect of the *Fc*γ*RIIB*-nt645 + 25A/G polymorphism being underestimated.

Additionally, the biological function of *Fc*γ*RIIB*-nt645 + 25A/G in the immune response has not yet been reported.

In the present study, our objectives were to investigate the possible association of the *Fc*γ*RIIB*-nt645 + 25A/G polymorphism with severity of periodontitis and with the levels of serum IgG against *P. gingivalis* in patients with periodontitis. Moreover, to find a functional effect of the *Fc*γ*RIIB*-nt645 + 25A/G polymorphism, we compared the expression levels of FcγRIIb on B lymphocytes in healthy subjects.

Material and methods

Patients with periodontitis

In this study, 32 Japanese patients with chronic periodontitis (11 men and 21 women; mean age, 40.6 years; age range: 26–59 years) were referred to the Periodontics Clinic at Niigata University Medical and Dental Hospital between April 2002 and March 2004. None of the participants had a history of, or current signs of, systemic disease, and had not used any medication within the 3 mo prior to this study. Informed consent was obtained from all participants via a signed form previously reviewed and approved by the Ethical Committee for the Use of Human Subjects in Research, Niigata University Faculty of Dentistry.

Clinical assessments of periodontitis

Patients were clinically evaluated at their first visit by several periodontists for the following conditions: number of teeth; probing pocket depth; and clinical attachment level (CAL). Probing pocket depth and CAL were assessed using a Williams probe at six sites around each tooth, namely the mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual and distolingual locations. Measurements were recorded to the nearest millimeter, and 0.5-mm measurement values were rounded down to the nearest 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 classified according to the Consensus Report of the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions (13) were selected for this study according to the following criteria: the presence of more than four diseased sites with probing pocket depth and CAL > 5 mm (not on the same tooth), together with radiographic evidence of bone loss. Smokers were identified using a standard questionnaire and excluded from the subjects. The clinical characteristics of patients are summarized in Table 1.

Preparation of sonicated *P. gingivalis* and 40-kDa OMP

P. gingivalis 381 was cultured in trypticase 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 (14).

Cultured cells of *P. gingivalis* were harvested by centrifugation, and sonicate was obtained using an ultrasonic homogenizer for 5 min. Recombinant 40-kDa OMP of *P. gingivalis* 381 was provided by Professor Y. Abiko (Department of Biochemistry and Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Japan) (15).

Measurement of serum IgG levels

Peripheral venous blood samples were obtained from all subjects by venipuncture. After coagulation, serum was collected by centrifugation at 1500 *g* for 20 min and stored at −20°C until used. The total IgG and subclass IgGs in serum that were specific for sonicated *P. gingivalis* and for 40-kDa OMP were determined by ELISA (16). Briefly, each of the 96-well flat-bottomed microtiter plates was coated, for 1 h at 37°C, with a capture monoclonal antibody for human IgG, 50 μL of sonicated *P. gingivalis* or 40-kDa OMP (1 μg/mL) in 50 mM sodium carbonate coating buffer (pH 9.6). After removal of the antigen solution, 200 μL of blocking reagent was added to each well for 20 min at room temperature. After washing three times 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 then washed three times with PBST and incubated, for 30 min at room temperature, with 50 μL of horseradish peroxidase-conjugated sheep anti-human IgG, IgG1, IgG2, IgG3 or IgG4 (1:1000 dilution) in PBST. After washing three times with PBST, color development was performed, for 10 min at 37°C, by the addition of 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 and stopped by the addition of 50 μL of 2 M H_2SO_4. The absorbance at 450 nm (OD 450 nm) was measured using 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 concentrations were calculated by relating the OD values (obtained by ELISA) from each serum sample from subjects to those of a reference serum and were 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 to 40-kDa OMP.

Determination of *FcγRIIB* genotypes

Genomic DNA was isolated from peripheral blood from patients and healthy subjects (Easy-DNA Kit; Invitrogen, San Diego, CA, USA) and stored at 4°C until genotyping was performed. The PCR methods used to amplify *FcγRIIB*, and later *FcγRIIB*-exon 4 with its flanking region, were as described previously (8). Briefly, because *FcγRIIA* and *FcγRIIC* genes were both highly similar to the *FcγRIIB* gene, we first performed *FcγRIIB*-specific PCR with primers specific for introns 3 and 6. After purification of the *FcγRIIB*-specific fragment, PCR with primers specific for exon 4 and its flanking region was performed using the purified *FcγRIIB* fragment as a template. PCR for *FcγRIIB*-exon 4 and its flanking region was performed in a 25-μL reaction mixture containing 0.5 U of Ex *Taq*™ (Takara Bio, Otsu, Japan) and 30 ng of the purified *FcγRIIB* PCR product, as follows: denaturation at 94°C for 5 min, 35 cycles of amplification (94°C for 30 s, 60°C for 30 s and 72°C for 30 s) and a final extension at 72°C for 5 min. The nucleotide sequences of these amplified fragments were determined using the ABI PRISM Big Dye Terminator Cycle Sequencing Kits and the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Table 1. Clinical characteristics and distributions of the FcγRIIB-nt645 + 25A/G genotype in subjects

Characteristic	Patients with periodontitis included in the analysis of serum antibody levels (n = 32)	Healthy subjects included in the analysis of the expression levels of FcγRIIb (n = 19)
Gender (male/female)	11/21	8/11
Age (years)	40.6 ± 9.2 (26–59)	32.7 ± 6.1* (27–49)
Number of teeth	26.7 ± 2.8	27.8 ± 1.8
FcγRIIB-nt645 + 25A/G genotypes (AA/AG/GG)	8/17/7 [25/53/22]	9/7/3 [47/37/16]
FcγRIIB-nt645 + 25A/G allele frequency (A/G)	0.52/0.48	0.66/0.34

Results are given as n, mean ± standard deviation (range) or n [%], unless stated otherwise.
*Statistically significant difference (p = 0.002, Mann–Whitney U-test).

Expression levels of FcγRIIb on peripheral B lymphocytes

The expression levels of FcγRIIb on peripheral B lymphocytes were compared among *FcγRIIB*-nt645 + 25A/G genotypes in 19 healthy Japanese volunteers. These volunteers had no clinical attachment loss, a probing pocket depth of ≥ 4 mm, alveolar bone loss ≥ 15%, no history of, nor present signs of, systemic disease and had not used any medication within the 3 mo prior to this study. Present smokers were excluded. As monoclonal antibody 41H.16 is specific to both human *FcγRIIb* and to an allele of the *FcγRIIa*-131R/H polymorphism, R131 (17,18), only *FcγRIIA*-131H/H homozygotes were included in this experiment. The *FcγRIIA*-131R/H genotype was determined by means of allele-specific PCR, as previously described (19).

Five millilitres of EDTA-anticoagulated peripheral blood was obtained from each subject. Erythrocytes were lysed by incubation with ammonium chloride. After washing with PBS, leukocytes were stained, first, with mouse monoclonal antibody 41H.16 (provided by Professor J. G. J. van de Winkel, UMC Utrecht, Utrecht, The Netherlands) and, second, with the fluorescein isothiocyanate (FITC) isomer I-conjugated $F(ab')_2$ fragment of rabbit polyclonal antibody specific to mouse IgG (Dako, Tokyo, Japan). After blocking with 10% mouse serum, leukocytes were stained with phycoerythrin-conjugated mouse monoclonal anti-human CD19 (eBioScience, San Diego, CA, USA). Phycoerythrin-conjugated mouse IgG2a and IgG1 were used as isotype controls. Double-stained leukocytes were resuspended in PBS and analyzed using a FACScan (BD Bioscience, San Jose, CA, USA). Dead cells were excluded by staining with 7-aminoactinomycin D. The mean fluorescence intensity of 41H.16 staining in $CD19^+$ cells was used as an indication of the expression level of FcγRIIb in peripheral B lymphocytes.

Statistical analysis

Differences between the *FcγRIIB*-nt645 + 25A/G genotypes in clinical measurements, serum antibody levels and the expression levels of FcγRIIb on peripheral B lymphocytes were assessed using the Kruskal–Wallis test or the Mann–Whitney *U*-test. Statistical significance was accepted at 5% ($p < 0.05$).

Results

Clinical characteristics and *FcγRIIB*-nt645+25A/G genotypes of patients with periodontitis

The clinical characteristics and *FcγRIIB*-nt645 + 25A/G genotypes of 32 patients with periodontitis are shown in Table 1.

As shown in Table 2, the numbers of teeth were significantly lower in *FcγRIIB*-nt645 + 25A carriers (AA and AG genotypes) compared with non-carriers (GG genotype) (Mann–Whitney *U*-test, $p = 0.02$). No significant difference was observed in gender, age and plaque-control records between the genotypes.

Clinical parameters of periodontitis in patients

The mean CAL in the *FcγRIIB*-nt645 + 25GG genotype was significantly lower than in the *FcγRIIB*-nt645 + 25AA genotype ($p = 0.003$) and in the *FcγRIIB*-nt645 + 25AG genotype ($p = 0.03$) (Fig. 1, Mann–Whitney *U*-test). Mean probing pocket depth and mean bone loss also tended to be lower in the *FcγRIIB*-nt645 + 25GG genotype, although the difference was not statistically significant. The heterozygotes showed intermediate values in mean probing pocket depth and mean CAL, although the mean bone loss in the *FcγRIIB*-nt645 + 25AG genotype was nearly the same as that in the *FcγRIIB*-nt645 + 25AA genotype.

Serum IgG levels nonspecific and specific to *P. gingivalis* antigens in patients with periodontitis

In a comparison of nonspecific serum IgG levels, as shown in Fig. 2, no significant difference in the levels of any IgG subclass was found between the *FcγRIIB*-nt645 + 25A/G genotypes.

When comparing the specific IgG levels produced against *P. gingivalis* sonicate (Fig. 3), significantly lower levels of IgG4 were found in subjects with the *FcγRIIB*-nt645 + 25AA genotype compared with GG homozygotes ($p = 0.03$).

The levels of specific IgG and IgG2 produced against the *P. gingivalis* 40-kDa OMP were significantly lower in patients with the *FcγRIIB*-nt645 + 25AA genotype than in patients with the *FcγRIIB*-nt645 + 25GG genotype (Fig. 4, $p = 0.02$ and 0.02, respectively).

Expression levels of FcγRIIb on peripheral B lymphocytes in healthy subjects

The clinical characteristics and *FcγRIIB*-nt645 + 25A/G genotypes of 19 healthy subjects are shown in Table 1. We found no significant difference of

Table 2. Comparison of clinical characteristics between FcγRIIB-nt645 + 25A/G genotypes in patients with periodontitis

	FcγRII-nt645 + 25AA ($n = 8$)	*FcγRIIB*-nt645 + 25AG ($n = 17$)	*FcγRIIB*-nt645 + 25GG ($n = 7$)
Gender (male/female)	4/4	6/11	1/6
Age (years)	41.6 ± 11.3	39.8 ± 8.9	41.6 ± 8.3
Number of teeth	26.3 ± 3.5	25.9 ± 2.4	28.9 ± 2.3*
Plaque control record (%)	57.2 ± 17.9	45.5 ± 20.9	58.9 ± 21.8

Results are given as *n* or as mean ± standard deviation.
No statistically significant difference was observed between *FcγRIIB*-nt645 + 25A/G genotypes (Fisher's exact test or Kruskal–Wallis test, $p > 0.05$).
*Difference between the *FcγRIIB*-nt645 + 25AA and *FcγRIIB*-nt645 + 25AG groups and the *FcγRIIB*-nt645 + 25GG group was significant (Mann–Whitney *U*-test, $p = 0.02$).

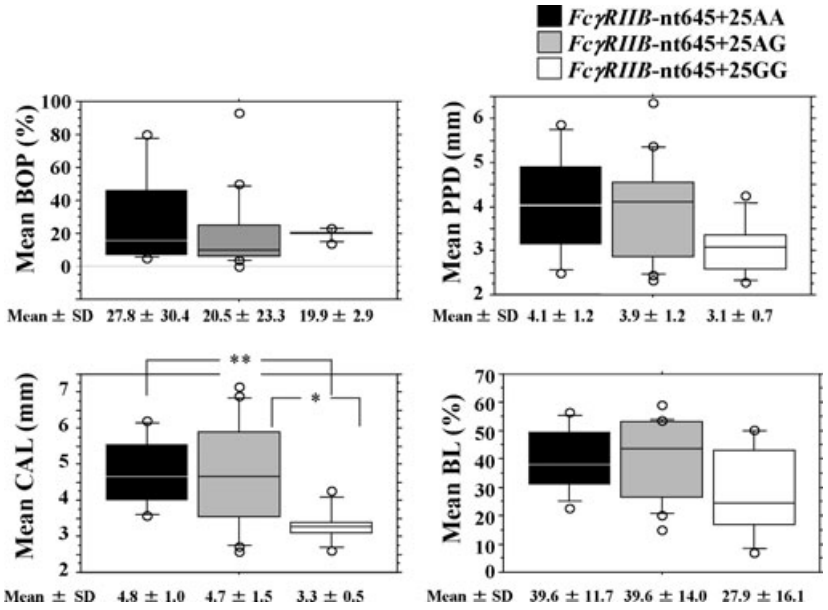

Fig. 1. FcγRIIB-nt645 + 25A/G genotypes and parameters of periodontitis. $n = 32$. $^{*}p < 0.05$, $^{**}p < 0.01$, Mann–Whitney *U*-test. BL, bone loss; BOP, bleeding on probing; CAL, clinical attachment level; PPD, periodontal probing depth; SD, standard deviation.

plaque control record and bleeding on probing between the genotypes in healthy subjects.

The levels of specific IgG2, IgG3 and IgG4 produced to *P. gingivalis* 40-kDa OMP were significantly lower in sera from healthy individuals than in patients with chronic periodontitis ($p = 0.0577$ for IgG1, $p = 0.0003$ for IgG2, $p = 0.0098$ for IgG3 and $p = 0.0416$ for IgG4, Mann–Whitney *U*-test), and there was no significant difference between *FcγRIIb*-nt645 + 25A/G genotypes ($p > 0.05$, Kruskal–Wallis test and Mann–Whitney *U*-test).

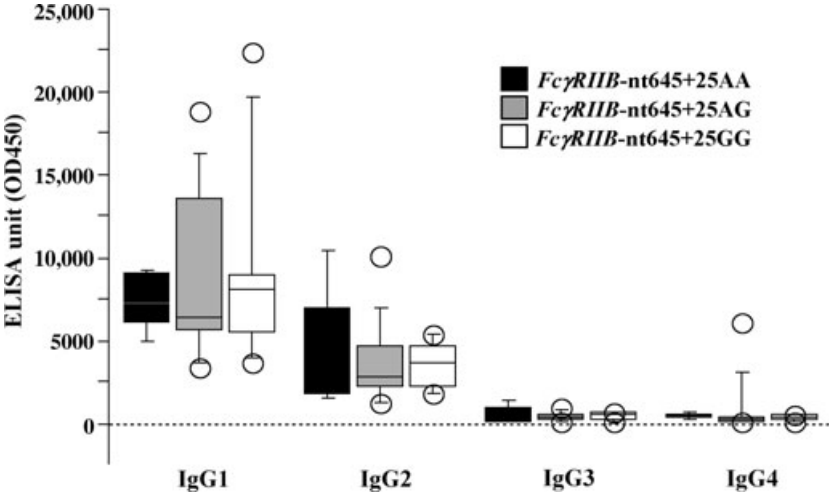

Fig. 2. FcγRIIB-nt645 + 25AG genotypes and nonspecific immunoglobulin G (IgG) in sera from patients with periodontitis. Sera from 32 patients with chronic periodontitis (eight with the *FcγRIIB*-nt645 + 25AA genotype, 17 with the *FcγRIIB*-nt645 + 25AG genotype and seven with the *FcγRIIB*-nt645 + 25GG genotype) were analyzed by ELISA. The absorbance value obtained at 450 nm (expressed as ELISA units) represents the IgG subclass concentration in the serum from each subject. No significant difference was observed between *FcγRIIB*-nt645 + 25A/G genotypes ($p > 0.05$, Mann–Whitney *U*-test).

Figure 5A is a typical scattergram of double staining of live cells with CD19, a marker for B lymphocytes, and 41H.16 antibody specific to human FcγRIIb and FcγRIIa-131R. As described in the Material and methods, all healthy subjects included in the FcγRIIb expression analysis had the *FcγRIIa*-131H/H genotype. Positivity for FcγRIIb in B lymphocytes ranged from 86 to 99% in the 19 healthy subjects. As shown in Figure 5B, the expression levels were significantly higher in the *FcγRIIB*-nt645 + 25AA genotype than in the *FcγRIIB*-nt645 + 25GG genotype ($p = 0.03$, Mann–Whitney *U*-test).

Discussion

In this study, patients with the *FcγRIIB*-nt645 + 25AA genotype had more severe periodontitis compared to those with the *FcγRIIB*-nt645 + 25GG genotype. In the same patients, the concentrations of specific antibody directed to *P. gingivalis* sonicate and to 40-kDa OMP were significantly lower in sera from subjects with the *FcγRIIB*-nt645 + 25AA genotype than in sera from subjects with the *FcγRIIB*-nt645 + 25GG genotype. Heterozygotes showed intermediate values both in the severity of periodontitis and in the concentrations of IgG specific to *P. gingivalis*. In healthy subjects, FcγRIIb was expressed at a significantly higher level on peripheral B lymphocytes from subjects with the *FcγRIIB*-nt645 + 25AA genotype than on peripheral B lymphocytes from subjects with the *FcγRIIB*-nt645 + 25GG genotype.

Previous studies have reported positive correlations between the severity of periodontitis and the levels of serum IgG against *P. gingivalis* (20,21). Patients with chronic periodontitis had significantly higher levels of serum IgG to *P. gingivalis* 40-kDa OMP compared with healthy controls (22). Consistent with the results of these reports, patients who participated in this study showed tendencies for weakly positive correlations between the severity of periodontitis and the concentrations of serum anti-*P. gingivalis* IgG, although the results

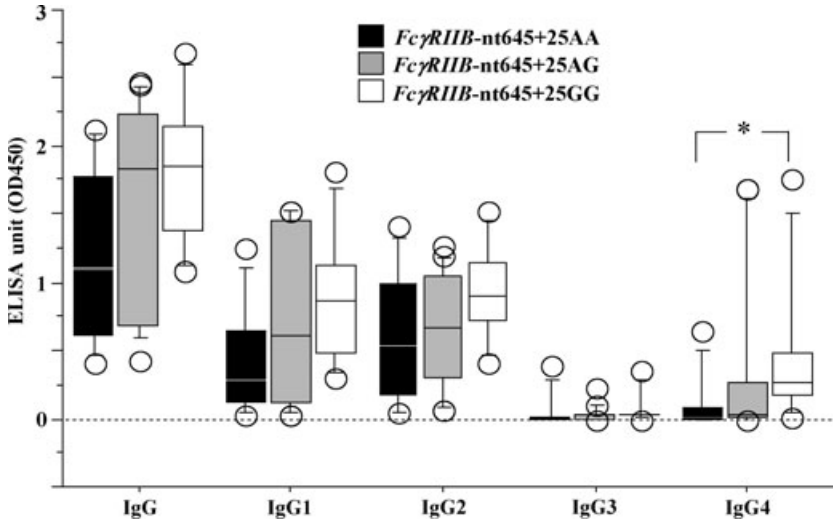

Fig. 3. *FcγRIIB*-nt645 + 25A/G genotypes and immunoglobulin G (IgG) against *Porphyromonas gingivalis* sonicate in sera from patients with periodontitis. Sera from 32 patients with chronic periodontitis (eight with the *FcγRIIB*-nt645 + 25AA genotype, 17 with the *FcγRIIB*-nt645 + AG genotype and seven with the *FcγRIIB*-nt645 + GG genotype) were analyzed by ELISA. The absorbance value obtained at 450 nm (expressed as ELISA units) represents IgG or each IgG subclass concentration reactive with *P. gingivalis* 381 sonicate in the serum from each subject. *$p < 0.05$, Mann–Whitney *U*-test.

were not statistically significant (for instance, $r = 0.261$, $p = 0.119$, for the correlation between mean CAL and the level of IgG against *P. gingivalis* 40-kDa OMP). However, patients with the *FcγRIIB*-nt645 + 25AA genotype had relatively lower levels of antibody against *P. gingivalis* and a higher severity of periodontitis.

Higher antibody levels in periodontitis patients with the *FcγRIIB*-nt645 + 25GG genotype may be caused by weaker inhibition of leukocyte activation and higher antibody production against periodontopathic bacteria. Impaired or weaker FcγRIIb function might induce stronger protection against infection. FcγRIIb-deficient mice were resistant to the manifestations of severe disease after infection with *Plasmodium chabaudi* (23). Human *FcγRIIB*-232T homozygosity was associated with protection against malaria and susceptibility to systemic lupus erythematosus in an East African population (24).

In a previous study, nonspecific total IgG and specific IgG levels to *P. gingivalis* 40-kDa OMP were significantly lower in sera from age-matched healthy subjects than in those from patients with chronic periodontitis (22). Consistent with the results of that report, in the present study, sera from healthy individuals showed significantly lower levels of IgG2, IgG3 and IgG4 specific to *P. gingivalis* 40-kDa OMP compared to those from patients with chronic periodontitis. There was no significant difference in the levels of specific IgG to *P. gingivalis* 40-kDa OMP among *FcγRIIb*-nt645 + 25A/G genotypes in healthy subjects. FcγRIIb has been reported to function as a negative regulator of antibody production in response to IgG immune complexes. In healthy subjects, the amounts of immune complexes might be too low to show any significant differences of specific antibody levels among *FcγRIIb*-nt645 + 25A/G genotypes.

Unfortunately, we could not obtain any subgingival microbial data in this study. The frequency of *P. gingivalis* in subgingival plaque from Japanese patients with chronic periodontitis has been reported to be 72% (25) and to be 50–60% in Japanese subjects with a mean probing pocket depth of 4.6 mm (26). A significant association was found between the detection of *P. gingivalis* and probing pocket depth (26). These facts would suggest that most

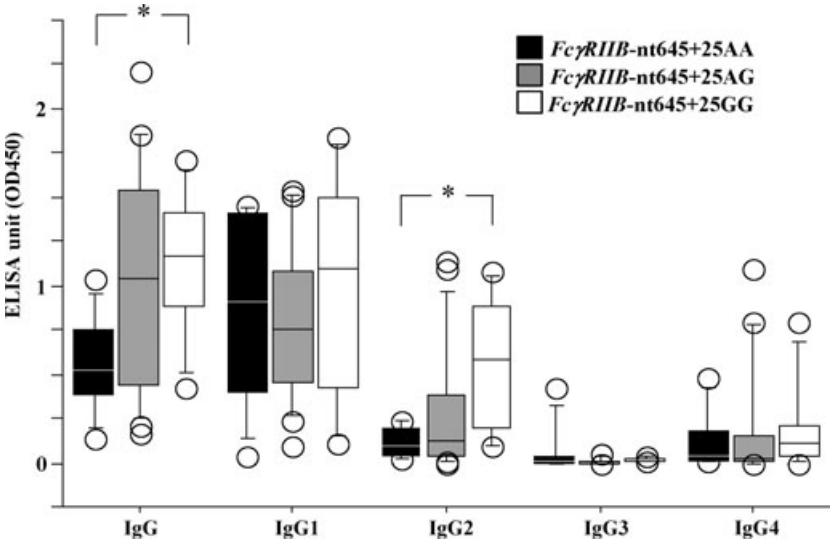

Fig. 4. *FcγRIIB*-nt645 + 25A/G genotypes and immunoglobulin G (IgG) against *Porphyromonas gingivalis* 40-kDa outer membrane protein (OMP) in sera from patients with periodontitis. Sera from 32 patients with chronic periodontitis (eight with the *FcγRIIB*-nt645 + 25AA genotype, 17 with the *FcγRIIB*-nt645 + AG genotype and seven with the *FcγRIIB*-nt645 + GG genotype) were analyzed by ELISA. The absorbance value obtained at 450 nm (expressed as ELISA units) represents IgG or each IgG subclass concentration reactive with *P. gingivalis* 40-kDa OMP in the serum from each subject. *$p < 0.05$, Mann–Whitney *U*-test.

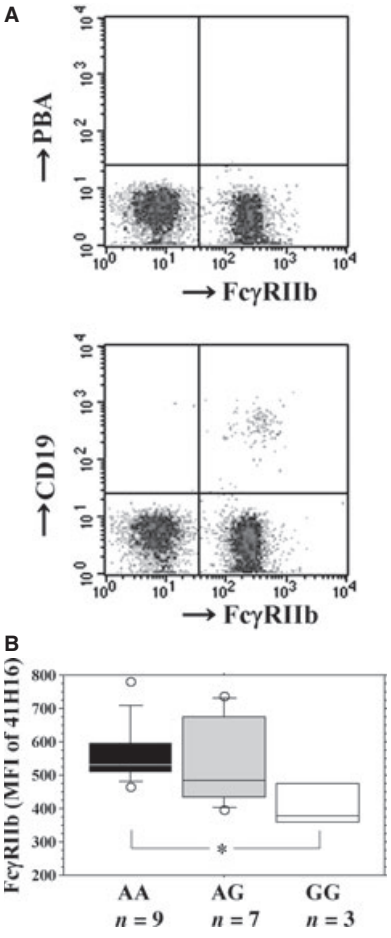

Fig. 5. FcγRIIB-nt645 + 25A/G genotypes and FcγRIIb expression levels in peripheral B lymphocytes from healthy subjects. Peripheral leukocytes were obtained from 19 healthy Japanese subjects with the *FcγRIIA*-131H/H genotype. (A) A typical scattergram of live cells double stained with a marker for B lymphocytes (CD19) and 41H.16 antibody (specific to human FcγRIIb and FcγRIIa-131R). (B) Mean fluorescence intensity of 41H.16 staining in $CD19^+$ cells were compared between *FcγRIIB*-nt645 + 25A/G genotypes. $*p < 0.05$, Mann–Whitney *U*-test. MFI, mean fluorescence intensity; PBA, phosphate buffered saline with 1% bovine serum albumin.

patients in this study had *P. gingivalis* in their subgingival microflora, although further studies are needed to confirm that antibody production in subjects with the *FcγRIIB*-nt645 + 25AA genotype would be lower in spite of the presence of *P. gingivalis*.

In this study, patients with periodontitis had more than four sites of disease with a probing pocket depth and CAL of > 5 mm, not on the same tooth, together with radiographic evidence of bone loss. This criterion might indicate more severe periodontitis than that of 'severe periodontitis' proposed by the United States Centers for Disease Control and Prevention Working Group for Use in Population-Based Surveillance of Periodontitis, which was defined as having more than two interproximal sites with a CAL of ≥ 6 mm, not on the same tooth (27).

In our previous study on sera from pregnant Japanese women, a lower level of IgG against *P. gingivalis* was associated with a higher prevalence of adverse pregnancy outcomes (28), which suggests that a lower humoral response may lead to weaker protection against infection, in agreement with the results from the present study. However, in postpartum Japanese women, preterm birth was associated with *FcγRIIB*-nt645 + 25A, and severity of periodontitis was associated with *FcγRIIB*-nt645 + 25G (12). This inconsistency may result from the differences in severity of periodontitis, the age of the subjects or characteristics of the antibody response during pregnancy. The degree of periodontitis was low in postpartum women and they were significantly younger than the patients with periodontitis in the present study.

We previously reported that the *FcγRIIB* polymorphism I232T was associated with the prevalence of aggressive periodontitis (8) and with lower levels of IgG2 against *P. gingivalis* 40-kDa OMP in chronic periodontitis but not with periodontal clinical parameters (11). *FcγRIIB*-I232T is located in exon 5, the transmembrane region, and the polymorphism changes the affinity of the FcγRIIb receptor to lipid rafts (9,10,29). On the other hand, *FcγRIIB*-nt645 + 25A/G is located in intron 4, and may change the expression level of this receptor, as suggested in this study. We have found significant linkage disequilibrium between *FcγRIIB*-I232T and *FcγRIIB*-nt645 + 25A/G (12). When comparing the results of a previous study (11) with those of the present study, the association with severity of chronic periodontitis and IgG levels against *P. gingivalis* seems stronger for the *FcγRIIB*-nt645 + 25A/G polymorphism than for the *FcγRIIB*-I232T polymorphism.

The functional difference between *FcγRIIB*-nt645 + 25AA and *FcγRIIB*-nt645 + 25GG may be caused by the difference in the expression levels of the receptor on B lymphocytes and presumably on other leukocytes. Two promoter haplotypes have been reported in human *FcγRIIB*: the common -386G:-120T variant and the less common -386C:-120A variant (30,31). A study by Su *et al*. (31) has shown the -386C:-120A variant to result in increased expression of FcγRIIb on monocytes, neutrophils and myeloid dendritic cells. However, we did not find this promoter polymorphism in pregnant Japanese women (12). Comparison of expression levels and nucleotide sequences of mRNA between *FcγRIIB*-nt645 + 25A/G genotypes will be needed to clarify whether the polymorphism affects the quantitative regulation of transcription or alternative splicing.

We used recombinant 40-kDa OMP as a specific antigen of *P. gingivalis* (32); 40-kDa OMP is a key virulence factor for co-aggregation and is found on many strains of *P. gingivalis* (33,34). 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 (35,36) and to have bactericidal and opsonic activities (36–39). Patients with periodontitis showed significantly higher serum IgG responses to the 40-kDa OMP than did subjects of the control group (22). The *FcγRIIB*-nt645 + 25AA genotype was associated with both lower levels of antibody specific to *P. gingivalis* 40-kDa OMP and higher severity of periodontitis. These results suggest that antibodies specific to *P. gingivalis* 40-kDa OMP may act protectively against periodontitis *in vivo*.

The total IgG concentrations in sera did not differ between the *FcγRIIB*-nt645 + 25A/G genotypes. The inhibitory function of FcγRIIb in B-cell activation is physically mediated by

B-cell receptor ligation via immune complexes. Thus, one possible explanation is that the functional difference of *FcγRIIB*-nt645 + 25AA and *FcγRIIB*-nt645 + 25GG genotypes could be invested in total nonspecific antibody responses *in vivo*.

Younger populations, without periodontitis, may contain individuals who will develop the disease in future. When we adopt young and healthy subjects as a control group, the risk to carry a genotype in development of the disease may be underestimated. In our previous study, in which we found no significant association between the *FcγRIIB*-nt645 + 25A/G polymorphism and periodontitis (8), the mean ages were 32 years for subjects with aggressive periodontitis, 50 years for subjects with chronic periodontitis and 26 years for healthy controls. If we had adopted age-matched controls, it may have been possible to find a significant association of the *FcγRIIB*-nt645 + 25 A/G polymorphism with the prevalence of aggressive or chronic periodontitis.

Additionally, in a previous study (8), the *FcγRIIB*-nt645 + 25A/G allele frequencies were 0.44/0.56 for patients with chronic periodontitis (n = 72) and 0.49/0.51 for healthy contols (n = 72). In the present study, the allele frequencies were, respectively, 0.52/0.48 for patients (n = 32) and 0.66/0.34 for healthy subjects (n = 19). There was no statistically significant difference of genotype distribution and/or allele frequency between patients and healthy subjects in the previous study and in the present study. However, in both studies, the relative frequencies of the A allele were higher in healthy subjects. These results might suggest that the *FcγRIIB*-nt645 + 25A/G polymorphism has different effects on the initiation of periodontitis depending on the severity of the disease. Investigations in large, age-matched populations will be needed to evaluate the contribution of the *FcγRIIB*-nt645 + 25A/G polymorphism to the occurrence and progression of periodontitis.

We tried to examine FcγRIIb expression levels on peripheral leukocytes preliminary in several patients with chronic periodontitis. However, the interindividual and day-to-day deviations in expression were greater than in healthy subjects and therefore we will need a large number of patients to reach a valid conclusion in the investigation. Additionally, the expression of FcγRIIb can be affected by many other factors as a result of inflammation and infection in patients. It has been reported that the expression level of FcγRIIb is up-regulated or down-regulated by cytokines, treatment with intravenous immunoglobulin (IVIG), complement, phorbol myristate acetate, etc. (40,41). For example, stimulation of B lymphocytes with interleukin-4 (IL-4) decreases the expression of *FcγRIIB* mRNA and the cell-surface expression of FcγRIIb (42), whereas interferon-γ (IFN-γ) increases the expression of *FcγRIIB* mRNA and the cell-surface expression of FcγRIIb in B lymphocytes stimulated with lipopolysaccharide (43). On the other hand, in monocytes, IL-4 increases the expression of *FcγRIIb*, and IFN-γ decreases it (44,45). As shown in the present study, a significant difference in the expression of FcγRIIb among the *FcγRIIB*-nt645 + 25A/G genotypes in healthy individuals suggests that the FcγRIIB-nt645 + 25A/G genotype affects FcγRIIb expression level even in stable, nonstimulated condition. However, *in vitro* studies will be needed to elucidate the difference of FcγRIIb expression levels during stimulation such as antibodies, antigens, bacterial products and cytokines.

In conclusion, the higher expression levels of FcγRIIb in subjects with the *FcγRIIB*-nt645 + 25AA genotype may induce the production of a lower level of IgG against *P. gingivalis* and therefore cause more severe 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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