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Rapid Communication

Association between the *Porphyromonas gingivalis fimA* type II genotype and the nutritional intake of elderly women

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The red complex bacteria *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), and *Treponema denticola* (*T. denticola*) are major causative agents of periodontitis and are highly associated with its severity. *P. gingivalis* exhibits a strong potential for adhesion and easily forms a biofilm. Its *fimA* gene can be classified by nucleotide sequence into six types. The *P. gingivalis* strains that possess type II *fimA* genotype are predominant in periodontitis patients. Here we investigated associations among the *P. gingivalis fimA* genotypes, periodontal disease infection in elderly women, and nutritional intake. We obtained samples from the participants' teeth and performed PCR using the primer specific for *P. gingivalis fimA* gene as a template and identified the bacteria through amplification of the 16S rRNA gene region. We amplified the *fimA* gene using primers specific to the six *fimA* genotypes. Dietary information over the previous 1−2 months was obtained via a questionnaire. *P. gingivalis* was detected in 97 % of the participants, in 49 % of which, the *P. gingivalis fimA* genotype was type II. Further, participants with the type II genotype had higher intakes of vitamin D, niacin, vitamin B_{12}, and fishery products and higher animal protein ratio in their diet than those with the other genotypes. The participants' protein/energy ratio was within the recommended levels, but their animal protein ratio was higher than that of people of the same age, and particularly high in those with the type II *fimA* genotype. The differences in intake suggest the presence of an association between nutritional intake and *P. gingivalis fimA* genotype, although determining the exact reason for this shall require further investigation.

Key words: *Porphyromonas gingivalis*; elderly women; *fimA* genotype; nutritional intake

1 Introduction

Periodontal disease is a chronic inflammation of the gums caused by pathogenic bacteria. In Japan, the prevalence of periodontal disease increases with age, affecting 53 % of the population aged 65−70 years and 62 % of those aged ≥ 70 years.[1] Further, the proportion of people aged ≥ 70 years with > 20 teeth is 37.1 %, which is lower than that for the other age groups.[2] Periodontal disease not only causes the loss of teeth but is also associated with systemic diseases, such as circulatory disease and diabetes.[3] Therefore, the prevention and treatment of periodontal disease are crucial. Bacteria known as the red complex species, namely *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), and *Treponema denticola* (*T. denticola*), are the major causative agents of periodontitis and are highly associated with its severity.[4] *P. gingivalis* exhibits a strong potential for adhesion and easily forms a biofilm. The *P. gingivalis fimA* gene can be classified into six types (I, Ib, II, III, IV, and V) based on its nucleotide sequences.[5,6] *P. gingivalis* strains that possess type II *fimA* are the most predominant in patients with periodontitis.[7-9]

There have been many studies on the risk factors of periodontal disease, which report smoking and obesity as potential risk factors.[10-14] It has also been reported that calcium, foods with lactic acid bacteria, dietary fiber, and n-3 fatty acids are associated with periodontal disease.[15-19] Moreover, female sex hormones are among the potential factors modifying the rate of progression of periodontal disease.[20,21] However, very few studies have investigated periodontal disease infection in elderly women who attended a lecture on health and its relationship with the genotypes of *P. gingivalis fimA*, but none have examined the association between these genotypes and nutritional intake. In this study, we investigated associations between

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periodontal disease infection in elderly women community residents, the genotypes of *P. gingivalis fimA*, and the women's nutritional intake.

2 Material and Methods

2.1 Participants

The participants were 35 female (aged 69.1 ± 4.7 years) general residents who participated in lecture events for the prevention of life-style related diseases held at Osaka Prefecture University. Lecture events were held twice a year. In this lecture event, lectures were given on diet and exercise for prevention of life-style related diseases. Elderly women who need nursing care and support were excluded. In addition, those who are being treated at a dental office were excluded. Their masticatory performance was at the level appropriate for a normal diet. Their masticatory performance was evaluated using chewing gum (Masticatory Performance Evaluating Gum XYLITOL, LOTTE). We conducted this study with the approval of the Ethical Review Board of the Osaka Prefecture University's Graduate School of Comprehensive Rehabilitation.

2.2 Plaque sampling and genomic DNA extraction

Dental plaque was collected from the participants' erupted teeth and subgingival with a sterile toothbrush for 1 min. The plaque adhering to the brush was removed by washing several times in a test tube of sterile distilled water. The plaque was collected by centrifugation at 1,600 × g for 20 min; the supernatant was discarded and the resultant pellets were stored at −20 °C until the DNA extraction.[22] The genomic DNA of each sample was extracted using the Wizard Genome DNA Purification Kit (Promega), and the samples were stored at −20 °C until use.

2.3 Detection of periodontal pathogens

The polymerase chain reaction (PCR) primers for detecting the bacterial species used in this study are listed in Table 1. PCR was initially performed with broad-range eubacterial primers based on the bacterial 16S ribosomal-RNA gene.[23] All primers were purchased from Invitrogen, Japan. The PCR reaction mixture included 0.25 U KOD FX Neo polymerase (TOYOBO), 0.2 mM deoxynucleotide triphosphates (dNTPs), polymerase buffer, 1 mM primers, and 30 ng of DNA solution from the plaque as the template DNA in a total volume of 25 μL. The samples were preheated at 95 °C for 2 minutes followed by 25 cycles of amplification under the following conditions: denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and elongation at 68 °C for 1 min using a T100 thermal cycler (Bio-Rad). The 25 cycles were followed by elongation at 68 °C for 5 minutes. The PCR products were confirmed by 1 % agarose gel electrophoresis and then purified using the Gel/PCR^{TM} DNA Isolation System (Viogene) in a unique final solution. A second nested PCR was performed with specific primers for periodontal pathogens designed on the basis of the 16S ribosomal-RNA gene (Table 1), reported previously.[24] The PCR reaction mixture included 0.25 U KOD FX Neo polymerase, 0.2 mM dNTPs, polymerase buffer, 1 mM primers, and 2 μL of purified first PCR products as a template DNA in a total volume of 25 μL. The PCR conditions were the same as previously described for the broad-range eubacterial primers except for the elongation time, which was 30 s. Subsequently, 5 μL of the PCR products obtained were mixed with 1 μL of Ez-Vision One (AMRESCO) and electrophoresed through 2 % agarose gel. The pathogen-specific bands were visualized under a UV light transilluminator.

2.4 PCR for the genotype analysis of P. gingivalis fimA

Table 2 shows the PCR primers for the genotype analysis of the *P. gingivalis fimA* gene as described in previous reports.[25] All primers were purchased from Invitrogen, Japan. The PCR reaction mixture included 0.25 U KOD FX Neo polymerase, 0.2 mM dNTPs, polymerase buffer, 1 mM primers, and 30 ng of DNA solution from the plaque as the template DNA in a total volume of 25 μL. Samples were denatured at 95 °C for 5 min, followed by 35 cycles of

Table 1 Primer sets for detection of specific periodontal pathogen.

Primer	Sequence	Amplified size (bp)
Eu-16S-S	5'- GAG TTT GAT CCT GGC TCA G -3'	
Eu-16S-AS	5'- AGA AAG GAG GTG ATC CAG CC -3'	~1,500
Pg-16S- S	5'- AGG CAG CTT GCC ATA CTG CG -3'	
Pg-16S- AS	5'- ACT GTT AGC AAC TAC CGA TGT -3'	404
Tf-16S-S	5'- GCG TAT GTA ACC TGC CCG CA -3'	
Tf-16S-AS	5'- TGC TTC AGT GTC AGT TAT ACC T -3'	641
Td-16S-S	5'- TAA TAC CGA ATG TGC TCA TTT ACA T -3'	
Td-16S-AS	5'- TCA AAG AAG CAT TCC CTC TTC TTC TTA -3'	316

denaturation at 94 ˚C for 30 s, annealing at 58 ˚C for 30 s, and extension at 72 ˚C for 30 s, with a final cycle of 72 ˚C for 7 min using the T100 thermal cycler. 5 μL of the PCR products obtained were mixed with 1 μL of Ez-Vision One and electrophoresed through 2 % agarose gel, with the pathogen-specific bands visualized under a UV light transilluminator.

2.5 Physical measurements

We used a body composition meter (BC118-D, TANITA) to measure the participants' body composition. Body mass index (BMI) was calculated by the standard formula: BMI = weight (kg)/height (m^2). BMI was divided into three categories: underweight (< 18.5 kg/m^2), normal (18.5−24.9 kg/m^2), and obese (≥ 25 kg/m^2).[26]

2.6 Nutritional intake

Nutritional intake was determined through the Food Frequency Questionnaire (FFQg version 3.0, Kenpaku Co., Ltd.), based on 29 food groups and 10 types of cooking. This was used to estimate the energy and nutrient intakes of each participant during the previous 1−2 months.

2.7 Statistical analysis

The chi-square test was used to evaluate differences in the detection ratios of the periodontal pathogens. Independent *t*-tests were used for the comparisons of nutritional intake and body composition according to the genotype of *P. gingivalis fimA*, and a *p*-value of < 0.05 was considered statistically significant.

3 Results

Fig. 1 shows the results of the detection of periodontal pathogenic bacteria. *P. gingivalis* was detected in 34 (97 %) of the participants, *T. denticola* in 28 (80 %), and *T. forsythia* in all 35 participants. All three strains were detected in 27 (77 %) of the participants. Fig. 2 shows the proportion of each genotype of *P. gingivalis fimA* detected. The sample collected from one participant was of a very poor quality and was therefore excluded. The type II *fimA* genotype was detected in 16 of the remaining 33 participants with *P. gingivalis*; this was a significantly higher proportion than for any of the other *fimA* genotypes ($p < 0.001$)

Table 2 Primer sets for analysis of *fimA* genotype.

Primer	Sequence	Amplified size (bp)
fimA-1-S	5'- CTG TGT GTT TAT GGC AAA CTT C -3'	
fimA-1-AS	5'- AAC CCC GCT CCC TGT ATT CCG A -3'	392
fimA-1b-S	5'- CAG CAG AGC CAA AAA CAA TCG -3'	
fimA-1b-AS	5'- TGT CAG ATA ATT AGC GTC TGC -3'	271
fimA-2-S	5'- GCA TGA TGG TAC TCC TTT GA -3'	
fimA-2-AS	5'- CTG ACC AAC GAG AAC CCA CT -3'	292
fimA-3-S	5'- ATT ACA CCT ACA CAG GTG AGG C -3'	
fimA-3-AS	5'- AAC CCC GCT CCC TGT ATT CCG A -3'	247
fimA-4-S	5'- CTA TTC AGG TGC TAT TAC CCA A -3'	
fimA-4-AS	5'- AAC CCC GCT CCC TGT ATT CCG A -3'	251
fimA-5-S	5'- AAC AAC AGT CTC CTT GAC AGT G -3'	
fimA-5-AS	5'- TAT TGG GGG TCG AAC GTT ACT GTC -3'	462

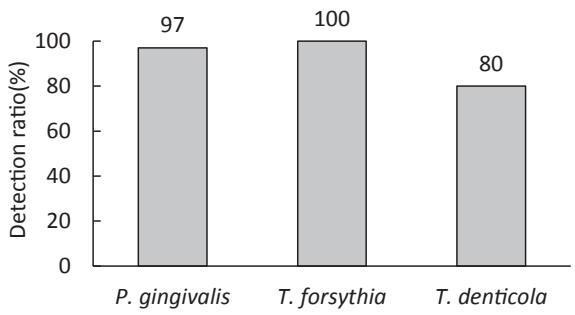
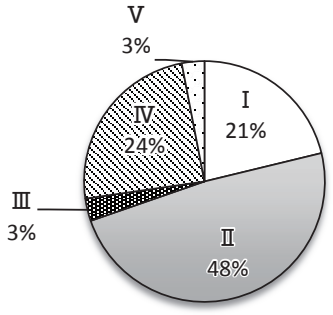


Fig. 1 Periodontopathic bacterial species detected in the participants' plaque.



Type Ib: not detected.

Fig. 2 Distribution of the five *P. gingivalis fimA* genotypes (n = 33).

Table 3 shows the physical characteristic data for the participants with *P. gingivalis*, comparing the 16 participants in which the type II *fimA* genotype was detected, with the remainder. There were no significant differences between the two groups. Overall, the mean BMI was 22.8 kg/m^2, with six participants having a BMI greater than 25 kg/m^2.

The participants' mean fat/energy ratio was within the range of 20−30 % energy indicated by the Dietary Reference Intakes (DRIs) for Japanese,[27] but the individual values for 19 (54 %) of the 35 participants exceeded 30 %. The protein/energy ratio was also within the range of 13−20 % energy indicated by the DRIs. Overall, the participants did not meet the DRI for dietary fiber, a factor for which an association with periodontal disease has been noted. The mean calcium intake reached the DRI value, but the intake of 10 (29 %) of the 35 participants was below the DRI value.

Table 4 presents the results of the nutritional intake

Table 3 Physical characteristics of the participants with *P. gingivalis*.

	P. gingivalis fimA type	
	II	I, III, IV, and V
	(n = 16)	(n = 17)
Age (years)	70.1 ± 4.5	68.1 ± 4.9
Height (cm)	151.3 ± 4.0	154.3 ± 3.8
Weight (kg)	54.2 ± 9.4	53.1 ± 7.5
BMI (kg/m^2)	23.7 ± 4.1	22.3 ± 2.8
Fat (%)	35.2 ± 6.9	30.7 ± 5.9

Values are mean ± SD. Type Ib was not detected.

Table 4 The nutritional intake of the participants with *P. gingivalis*, comparing those with the type II *fimA* genotype with the remainder.

	P. gingivalis fimA type	
	II	I, III, IV, and V
	(n = 16)	(n = 17)
Energy (kcal)	2045 ± 418	1912 ± 445
Protein/energy ratio (%energy)	16.0 ± 2.5	14.6 ± 1.4
Fat/energy ratio (%energy)	30.2 ± 4.0	29.4 ± 3.7
Carbohydrate/energy ratio (%energy)	53.7 ± 6.0	56.0 ± 4.8
Protein (g)	82.6 ± 23.4	70.3 ± 20.5
Animal protein ratio (%)	57.6 ± 8.4*	51.9 ± 6.1
Calcium (mg)	709 ± 185	681 ± 212
Phosphorus (mg)	1232 ± 331	1101 ± 310
Iron (mg)	9.0 ± 2.3	8.0 ± 2.2
Magnesium (mg)	294 ± 71	268 ± 75
Zinc (mg)	9.4 ± 2.4	8.3 ± 2.1
Copper (mg)	1.22 ± 0.24	1.13 ± 0.30
Retinol activity equivalents (μg)	710 ± 156	659 ± 169
Vitamin D (μg)	11.9 ± 4.9*	8.2 ± 3.5
α-Tocopherols (mg)	7.6 ± 1.7	6.9 ± 2.0
Vitamin K (μg)	264 ± 61	252 ± 67
Thiamin (mg)	1.12 ± 0.29	0.95 ± 0.26
Riboflavin (mg)	1.28 ± 0.35	1.16 ± 0.33
Niacin (mg)	19.4 ± 7.0*	14.9 ± 4.8
Vitamin B_6 (mg)	1.40 ± 0.37	1.18 ± 0.32
Vitamin B_{12} (μg)	10.5 ± 4.4*	7.3 ± 2.8
Vitamin C (mg)	125 ± 31	114 ± 33
Folate (μg)	347 ± 67	329 ± 82
Pantothenic acid (mg)	6.31 ± 1.50	5.77 ± 1.57
Dietary fiber (g)	16.0 ± 2.6	15.5 ± 4.2
Salt equivalent (g)	10.6 ± 2.3	10.5 ± 4.0
Cholesterol (mg)	366 ± 140	309 ± 115
Potassium (mg)	2864 ± 646	2602 ± 720
n-6 Fatty acids (g)	10.79 ± 3.16	10.43 ± 3.47
n-3 Fatty acids (g)	2.84 ± 0.94	2.30 ± 0.82

Values are mean ± SD. * $p < 0.05$. Type Ib was not detected.

analysis for the 33 participants with *P. gingivalis*, showing a comparison between the 16 participants with the type II *fimA* genotype and the remainder. Those with the type II genotype consumed a significantly higher ratio of animal protein to total protein ($p = 0.036$) and had significantly higher intakes of vitamin D ($p = 0.012$), niacin ($p = 0.046$), and vitamin B_{12} ($p = 0.019$). The protein/energy ratio was also higher in those with the type II genotype, but this did not achieve statistical significance ($p = 0.056$). There was no significant difference between the groups for any other nutrient. In the analysis of food groups, those with the type II genotype showed a greater intake of fishery products ($p = 0.012$).

4 Discussion

Elderly women examined in this study were participants who took part in lecture events for life-style related diseases prevention on their own will. Considering this fact, participants' awareness of health was not necessarily low. Nevertheless, 16S rRNA gene of *P. gingivalis* was detected in 97 % of the participants (Fig. 1), and the type II genotype of *fimA* gene in *P. gingivalis* was found in 48 % of those who *P. gingivalis* detected women (Fig. 2). It has often been reported that the type II genotype is predominant in patients with severe periodontal disease;[7] we speculated that the periodontal disease was possibly progressing in people infected with *P. gingivalis* with this genotype. The relationship between the genotype and the virulence have discussed in many reports from the pathogenic and molecular mechanistic point of view.[28-36] However, the relationship between the genotype and nutrient intakes was unknown till now. Thus, we examined this relationship for elderly women by using the food frequency questionnaire (FFQ) method.

Comparing of nutrient intakes between the participants with *fimA* type II genotype and with other types indicated that those with the type II genotype was significantly higher intakes of vitamin D, niacin, and vitamin B_{12} (Table 4). In the DRIs for Japanese, the tolerable upper intake level (UL) values were set for vitamin D and niacin. UL was set to prevent adverse health conditions that would be caused by an excessive intake of certain nutrients. UL value of vitamin D was 100 μg, and niacin was 250 mg (quantity as nicotinamide) and 60 mg (quantity as nicotinic acid), but the participants with *fimA* type II genotype did not reach UL values (vitamin D: 11.9 ± 4.9 μg, niacin: 19.4 ± 7.0 mg in Table 4). In the DRIs for Japanese, the protein/energy ratio has tentative dietary goal for preventing life-style related diseases (DG) value. DG value of protein/energy ratio was 13−20 % energy. The mean protein/energy ratio in participants of both groups met DG value (16.0 ± 2.5 % energy, 14.6 ± 1.4% energy in Table 4). Moreover, the animal protein ratio of the participants (54.6 ± 8.0 %) was higher than that of women with similar ages (60−69 years old: 50.7 %, 70 years and over: 49.5 %).[2] Notably, the animal protein ratio of the participants with the type II *fimA* genotype (57.6 ± 8.4 %) was particularly high. To date, what makes the difference of these nutrients between two groups of the *fimA* genotypes remains in this study. Further investigation is required for understanding the relationship between these nutrients and *fimA* genotypes.

A previous study reported that periodontal disease was more severe in women whose amount of daily calcium intake was less than 500 mg compared to 800 mg or more.[15] The calcium intake of both groups showed more than 500 mg and no significant difference between two groups (709 ± 185 mg, 681 ± 212 mg in Table 4). In addition, it is reported that benefits of higher intake of high-fiber foods on slowing periodontal disease progression are most evident in men aged 65 and older.[18] In this study, the intake of dietary fiber was almost equivalent for two groups (16.6 ± 2.6 g, 15.5 ± 4.2 g in Table 4). But, it is difficult to directly compare the value of the intake between previous studies and our present study because the diet survey method was different among these studies. The intake of dietary fiber of participants in this study were less than DG value (50−60 years old: ≥ 18 g, 70 years and over: ≥ 17 g), suggesting that it is desirable for both groups to increase the intake of the high-fiber foods. Furthermore, higher dietary intakes of n-3 fatty acids were associated with lower prevalence of periodontitis.[19] The n-3 fatty acids intake of the type II group (2.84 ± 0.94 g) was higher than other genotype group (2.30 ± 0.82 g) in the study (Table 4) though the difference between two groups was not significant, and both groups met the adequate intake (AI) value, which indicates the amount adequate to maintain a certain level of nutritional status, in the DRIs for Japanese. The result of these three nutrients was apparently inconsistent with previous reports described above because the type II genotype was mainly found in the patients with severe periodontitis. These reports were studied based on western diet for western people. This study was the result of Japanese food for Japanese. Therefore, there was also a possibility that out differences. For that reason, it may be necessary to consider diet style and racial differences.

There are some limitations to this study. First, a diet survey was based on a self-report. Second, there may have been selection bias because participant attended a program of their choice. To clarify the relationship between the nutrient intakes and the *fimA* genotypes, further study is required: for instance, whether the amount of these nutrient affect the growth of *P. gingivalis*, the biofilm formation and

colonization to the cells depending on the *fimA* genotypes *in vitro* and *in vivo*. Promotion of healthy nutrition and adequate physical activity may be additional factors to help prevent or delay the progression of periodontal disease.[37] A more detailed investigation into dietary habits will elucidate the association between the nutrient intakes and the genotypes of *P. gingivalis fimA* gene, by fulfilling a new study including various aged people with and without *P. gingivalis* infection.

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