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Evaluation of *RANK/RANKL/OPG* gene polymorphisms in aggressive periodontitis

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Background and Objective: Aggressive periodontitis (AgP) is a specific type of periodontal disease that is characterized by rapid attachment loss and bone destruction. While attempting to identify genetic polymorphisms associated with AgP, previous research has focused on candidate genes that may be involved in immune responses to microbial infections. In this study, the focus was on single nucleotide polymorphisms (SNPs) in the key mediators of osteoclast differentiation and activation, which involve receptor activator of nuclear factor-κB (*RANK*), RANK ligand (*RANKL*) and osteoprotegrin (*OPG*), in the Japanese population. The aim of this study was to evaluate the association of *RANK/RANKL/OPG* gene polymorphisms with AgP in the Japanese population.

Material and Methods: We examined 99 patients with AgP and 89 controls from the Japanese population to explore the possibility of *RANK/RANKL/OPG* loci as candidate regions associated with the disease. All exons and relevant exon–intron boundaries of these three candidate genes were amplified by polymerase chain reaction (PCR) using 19 primers, followed by direct sequencing. The polymorphisms were identified by comparing the sequences obtained from 48 subjects.

Results: We identified 27 SNPs in *RANK*, including 10 novel SNPs and seven SNPs each in both *RANKL* and *OPG*. A pairwise linkage disequilibrium analysis using the r^2 statistic showed that some SNP pairs from the three loci are in tight linkage disequilibrium.

Conclusion: An association analysis with allelotypes showed that SNPs identified in the *RANK/RANKL/OPG* genes have no significant association with AgP in the Japanese population.

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Aggressive periodontitis (AgP), previously classified as early-onset periodontitis (1,2), is a specific type of periodontal disease that is characterized by rapid attachment loss and bone destruction, resulting in tooth loss at early onset (< 35 years of age). Bacterial plaque, inflammatory factors and other environmental factors may play major roles in periodontal disease progression, but several studies demonstrated that genetic factors influence the susceptibility to AgP (3–11). Genetic variance obtained from twin studies indicated that some of the clinical characteristics of periodontitis may be influenced by genetic factors (12–14). Candidate genes that have been investigated as genetic markers of AgP include interleukin-1α (*IL-1α*), interleukin-1β (*IL-1β*), interleukin-1 receptor (*IL-1R*) and interleukin-1 receptor antagonist (*IL-1RN*) (15–19), tumour necrosis factor-α (*TNF-α*) (6,19,20), Fc-γ receptors (*Fc-γR*) (21), interleukin-4 (*IL-4*) (19,22), interleukin-10 (*IL-10*) (10,19,23), formylpeptide receptor (*FPR*) (24,25), collagen, epidermal growth factor (*EGF*), transforming growth factor (*TGF*), and so on (19). Among these genes, *IL-1* is known as the key regulator of the host responses to microbial infection; thus, it is considered to be an important factor in the pathogenesis of periodontal disease in general (15,16). Genetic polymorphism detected in the cluster of *IL-1* thus far has proved that *IL-1β* and *IL-1RN* may be associated with AgP (16,26–28).

It has also been reported that *Fc-γR* is associated with susceptibility to AgP and chronic periodontitis (21,29).

Recent advances in bone cell biology demonstrated the crucial role of the receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL)/osteoprotegrin (OPG) system in osteoclast differentiation and function. RANKL is a cytokine that belongs to the TNF family and is essential for the induction of osteoclastogenesis. Osteoblasts and bone marrow stromal cells produce this cytokine, and its signal is transduced by the specific receptor, RANK, which localizes on the cell surface of osteoclast progenitors. OPG is also a cytokine that belongs to the TNF family and is produced by osteoblasts and bone marrow stromal cells. OPG inhibits osteoclastogenesis by being involved in the competitive binding of RANK with RANKL. Thus, RANKL and OPG regulate bone resorption by exerting a positive or a negative control on the activation of RANK present on osteoclasts. The RANK/RANKL/OPG regulatory axis is also involved in inflammatory bone destruction induced by pro-inflammatory cytokines such as prostaglandin E_2 (PGE_2), *IL-1β*, *IL-6*, *IL-11* and TNF-α (30,31).

Several studies have demonstrated the functional association of RANKL and OPG with periodontal disease. In animal experiments, $CD4^+$ T cells stimulated by *Actinobacillus actinomycetemcomitans* induce RANKL production (3,32,33) and participate in alveolar bone destruction. Two studies that used samples obtained from humans with periodontal disease also demonstrated an elevated level of RANKL expression and a reduced level of OPG expression in the affected tissues when compared with those in healthy gingival tissues (34,35). Thus, the RANK/RANKL/OPG regulatory axis plays a major role in bone destruction during periodontitis, including AgP.

Although a considerable, positive association in the genetic markers of *RANK* and *OPG* has been observed in bone-destructive diseases, such as Paget's disease of bone (PDB) (36), familial expansile osteolysis (FEO) (37) and human osteoporosis (38), no such studies have been conducted on AgP. Therefore, in the present study we investigated single nucleotide polymorphisms (SNPs), which are the simplest mutation studied in genes, associated with *RANK*, *RANKL* and *OPG*. An association analysis with allelotypes showed that SNPs identified in the *RANK*, *RANKL* and *OPG* genes have no significant association with AgP in the Japanese population.

Material and methods

Subjects

A total of 99 Japanese patients with AgP (64 women and 35 men; mean age: 30.9 ± 6.2 years, range: 17–47 years) were recruited at the Department of Periodontology in Aichi-gakuin University from 2002 to 2004. The regionally matched control group comprised 89 periodontally healthy volunteers (33 women and 56 men; mean age: 42.9 ± 7.0 years, range: 34–63 years). The diagnostic criteria of AgP, based on Tonetti's study (1), were defined as follows: (i) age of onset of periodontitis < 35 years of age and (ii) attachment loss of ≥4 mm in at least four permanent teeth, including at least one first molar. Alveolar bone loss in all the AgP patients was assessed using full-mouth radiographs. In this study, 98 AgP patients had attachment loss of ≥4 mm in eight or more teeth. At the initial visit, 28 patients with periodontitis were older than 35 years; however, onset of periodontitis was confirmed at less than 35 years of age, based on dental history. Periodontally healthy control subjects were older than 35 years and had no attachment loss in any teeth; however, some control subjects had gingival pockets in a few teeth. The systemic health of all the participants was confirmed by general blood tests. The degree of periodontitis in all the participants was assessed by measuring periodontal pocket depth, clinical attachment level, bleeding on probing and degree of teeth mobility. Clinical characteristics of AgP patients and control subjects are summarized in Table 1.

Genomic DNA was extracted from whole peripheral blood using the Nucleon genomic DNA Extraction kit (Tepnel Life Sciences PLC, Manchester, UK). Written informed consent was obtained from all the participants. This study was approved by the ethical committees of the School of Dentistry, Aichi-gakuin University, and of the RIKEN Yokohama Institute.

Identification of SNPs

All sequence references from *RANK*, *RANKL* and *OPG* were obtained from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), with accession numbers NT_025028 for *RANK*, NT_024524 for *RANKL* and NT_008046 for *OPG*. Polymerase chain reaction (PCR) amplification was performed by using PCR primer sets designed to amplify all coding exons and exon–intron boundaries of the *RANK*, *RANKL* and *OPG* genes. The reaction medium of 20 μl volume contained 2.5 mM dNTPs, 2.5 mM Mg^{2+}, 50 pmol of each primer, 0.5 U of LA Taq or Ex Taq DNA polymerase

Table 1. Clinical parameters of patients with aggressive periodontitis (AgP) and of control subjects

Trait	AgP	Control
Number	99	89
Gender ratio (F/M)	64/35	33/56
Age (yr)	30.90 ± 6.2	42.90 ± 7.0
Number of present teeth	27.72 ± 2.82	27.62 ± 2.35
Mean PD (mm)	4.01 ± 1.33	1.94 ± 0.30
Mean CAL (mm)	4.55 ± 1.56	2.15 ± 0.36
Ratio of teeth PD ≥ 4 mm (%)	0.78 ± 0.24	0.09 ± 0.13
Ratio of teeth CAL ≥ 4 mm (%)	0.82 ± 0.20	0.19 ± 0.17
Ratio of teeth BOP (+) (%)	0.73 ± 0.29	0.14 ± 0.16
Ratio of teeth mobility ≥ 2° (%)	0.11 ± 0.16	0

Values represent mean ± standard deviation (SD).
BOP, bleeding on probing; CAL, clinical attachment level; PD, probing depth.

(Takara, Otsu, Japan) and 5 ng of genomic DNA. The PCR amplification was performed for 40 cycles, as follows: at 96°C for 10 s for denaturation, at 55°C–60°C for 10 s for annealing, and at 72°C for 1 min for extension. After purification, the amplified genomic DNA was sequenced by using a Big-Dye Terminator Cycle Sequencing Kit and an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Ten exons of the *RANK* gene were sequenced using 10 sequencing primers and two overlapped sequence primers for exons 5 and 9; five exons of the *RANKL* gene were sequenced, by using three sequencing primers and three overlapped sequence primers for the last exon, and five exons of the *OPG* gene were sequenced, by using four sequencing primers and three overlapped sequence primers for the last exon. PCR primer sets and sequencing primers are shown in Table 2. Polymorphisms were identified by using the SeqScape software (Applied Biosystems) and comparing sequences from 96 chromosomes from 24 Japanese patients with AgP and 24 control subjects. Both strands of the PCR genomic DNA were reamplified and resequenced in order to confirm the polymorphism.

Statistical analysis

The Fisher's exact test (two-sided *p*-value) was performed between the patients and control subjects for each allelic frequency. Statistical significance was determined at $p < 0.05$. Pairwise linkage disequilibrium (LD) was estimated as $D = x_{ij} - p_1p_2$, where x_{ij} is the frequency of haplotype A_1B_1 and p_1 and p_2 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A standardized LD coefficient, *r*, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where q_1 and q_2 are the frequencies of the other alleles at loci A and B, respectively. Lewontin's coefficient D′ is given by D/D_{max}, where $D_{max} = \min[q1p2, p1q2]$ when $D > 0$. Haplotype frequencies for multiple loci were estimated by the expectation maximization method. Computations were performed by using the SNPALyze software (Dynacom, Mobara, Japan).

Results

Molecular variants in *RANK*, *RANKL* and *OPG*

All variants were identified from 24 AgP patients and 24 controls. From the 60.95-kb genomic region of the complete *RANK* gene, we sequenced all 10 exons and the exon–intron boundaries of *RANK* (Fig. 1). We identified 27 SNPs: three were within the coding region, one was within the 5′ untranslated region, 23 were within introns and one was within the 5′ flanking region. Ten novel SNPs were detected among the 27 SNPs; two were located in exon 1 and exon 10. Among the SNPs in the coding region, two were synonymous substitutions and one was a nonsynonymous substitution. From the 33.85-kb genomic region of the *RANKL* gene, we sequenced five target exons and exon–intron boundaries. Seven SNPs were identified: two were within the coding region, two were within the 3′ untranslated region and three were within introns. The SNPs in the coding region

Table 2. Oligonucleotide sequence of receptor activator of nuclear factor-κB (RANK), *RANK* ligand (*RANKL*) and osteoprotegrin (*OPG*) for polymerase chain reaction (PCR) amplification and sequencing

Gene	Fragment ID	Forward primer	Reverse primer	Sequencing primer	Direction
RANK	1	cccagacatctggccacttt	ccgtccaactctggaagc	gcaaaccaggggagctt	F
	2	tgcagtccttcaggtaacag	gcagcaacaattccagtgtg	gcagcaacaattccagtgtg	R
	3	gaagttgcctatccagatgg	catcctggctaacatggtga	actgcattgtggcctcttct	F
	4	aagtgcgaggaggaacttgg	aaaaaccctccaagcataaaa	tgtcctggtgattcactctg	F
	5	ggactgaggagattaagctc	tgacagcatgcaaacattta	cgtggttccataactcactgc	F
	5			aagctgcaaccgtaagtcacag	R
	6	ttgctgaccgcaatctcaga	gttacttggtgagcaggaca	ctcacttgtgaccaccatca	R
	7	ggagaggaaaagtaggagtc	ccatatatgtgatgctgcagag	ccatatatgtgatgctgcagag	R
	8	acttgcggtggactctgttg	cctggaaatgccctgtaagc	acttgcggtggactctgttg	F
	9	gggttagctcctctccttgc	agaggcctaaggtgctttga	gaatccaaagccatcctg	R
	9			tgaagcactggctt	R
	10	tgttagggaccatgaggaac	acctcccagatgtgcaaaat	agggttacagtgtgg	F
RANKL	1	agacagctgaggatggcaag	agattcaaacccatcgtccg	tctcccttctggagtcactt	R
	2	aagtgtgccttgtcctgtgg	tgcatgaacgtccctgattg	aactgtgccttgtcctgtgg	F
	3,4	tcgttctctttcctgtgggc	agcagggctgtgtcaacttt	tcacctgaggatgaatggtc	R
	5	gccttacttctgctctgaac	ggccaatgattgagttccac	gcaccaagtacatc	F
	5			aatcagcatcgagg	F
	5			ctgcatttctcccagtaacc	R
OPG	1	aagttcagcgcgtaggaagc	tgagggcgttaatattctgagatg	gtggaggagacacaagcaca	R
	2	tgcaacccaagacgacaaag	tcaccaattcctccctcaaa	tgctatctgcattcctggtc	R
	3	gaggagaaactgccaaaggg	gtggcaaacttgacactgcc	gtggcaaacttgacactgcc	F
	4	ttcaaagccaggtctgatga	cgttaagggatctggaagcg	taagaccagccaacagaagc	R
	5	ccttagttcctcgacctca	cttacgtagcttggtgtctc	gtgtcacttaactccctctc	F
	5			tcaggcacttgaggctttc	R
	5			cagctatgctagag	F

All primer sequences are given in the 5′ to 3′ direction.
F, forward; R, reverse.

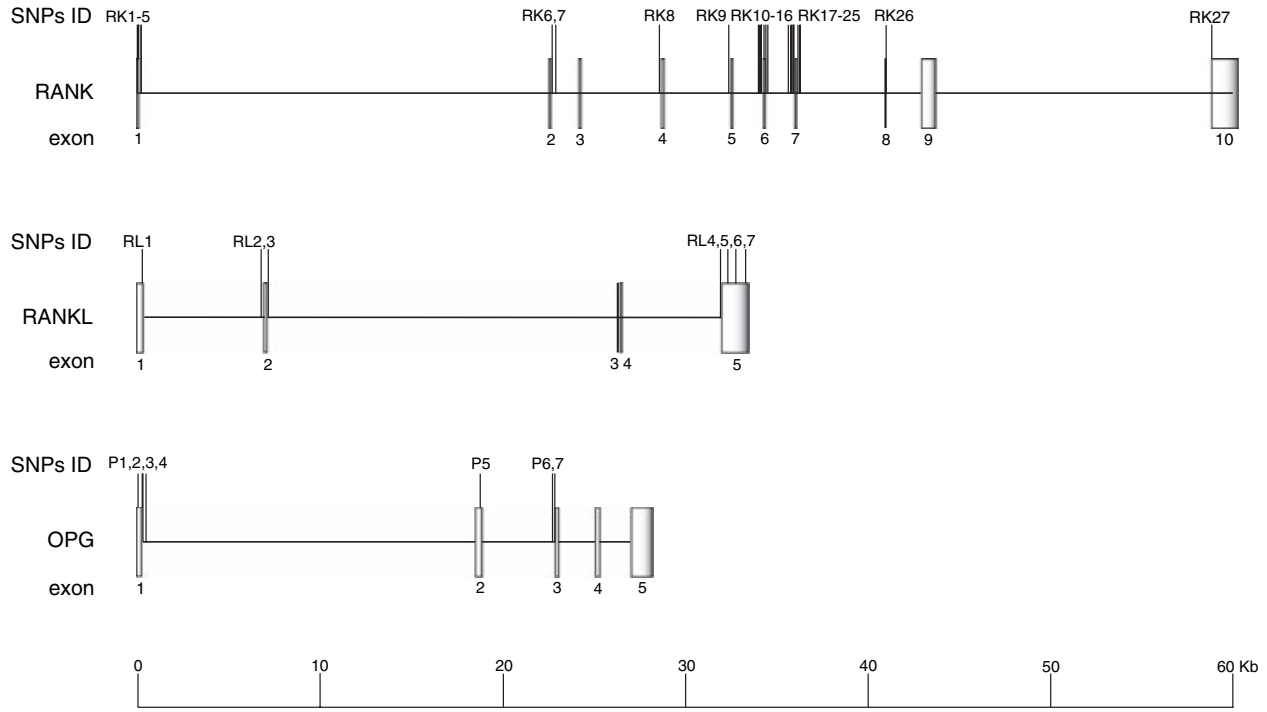

Fig. 1. Single nucleotide polymorphism (SNP) map of the genomic region containing the receptor activator of nuclear factor-κB (*RANK*), RANK ligand (*RANKL*) and osteoprotegrin (*OPG*) genes. Black rectangle, exons; horizontal lines, introns. SNPs are indicated above the lines according to number.

were classified as synonymous mutations. Five exons and exon–intron boundaries were sequenced within the 28.51-kb genomic region of the *OPG* gene. Two SNPs were detected in the coding region and identified as nonsynonymous: one was within the 5′ untranslated region and four were within introns; in all, seven SNPs were identified within the gene. Transition substitution was more prevalent than transversion substitution in all the three genes. No insertion/deletion polymorphism was detected from the sequenced subjects. SNPs with a minor allele frequency greater than 10% were defined as common SNPs. Table 3 shows that from the 96 chromosomes sequenced from 48 subjects, we identified 20 common SNPs from *RANK*, four common SNPs from *RANKL* and six common SNPs from *OPG*.

Pairwise LD in *RANK*, *RANKL* and *OPG*

The strength of LD for each SNP pair from each gene was measured using the r^2 and D' values. SNP pairs in high LD were measured as l D'l or $r^2 \geq 0.70$. In *RANK*, six subgroups of SNP (namely, RK1 and RK2; RK7 and RK16; RK9 and RK17; RK11 and RK21; RK12, RK13, RK14, RK15, and RK24; and RK18 and RK25) showed a tight LD with each other, as shown in Fig. 2. In *RANKL*, the major subgroups were RL1 and RL7; RL2 and RL3; and RL4, RL5 and RL6. From the *OPG* gene, we obtained the following two major subgroups: P2 and P6; and P3 and P7. SNPs without a tight LD were considered to be independent.

Allele frequency distributions in AgP patients and periodontally healthy controls

An additional 51 Japanese AgP patients and 41 controls were genotyped for the chosen SNP from each major subgroup of the three genes and for independent SNPs not in LD with others. In the *RANK* gene, association analysis was conducted using RK2, RK9, RK11, RK14, RK16 and RK18 and the independent SNPs, namely, RK3, RK4, RK6, RK8, RK10, RK19, RK20, RK26 and RK27. The *RANKL* association analysis used RL1, RL2 and RL4 and no independent SNPs. For *OPG*, the P2 and P3 SNPs were chosen, and the independent SNPs were P1, P4 and P5. Therefore, P5, a nonsynonymous SNP, was included for analysis, although the allele frequency was less than 10%. The distribution of genotypes in AgP patients and controls were in agreement with the Hardy–Weinberg equilibrium expectations. No significant difference in the SNP frequency distribution was observed between AgP patients and controls in *RANK*, *RANKL* and *OPG* (Table 4).

Discussion

The frequency of genetic alleles showed a tendency to differ among the different populations that were examined. In our study, we collected and examined samples from the Japanese population that is considered a homogenous population based on genetic variance studies. Therefore, the difficulty of distinguishing different ethnic groups within the population was eliminated. Furthermore, we selectively recruited control subjects who were older than

Table 3. Sequence polymorphisms in receptor activator of nuclear factor-κB (*RANK*), RANK ligand (*RANKL*) and osteoprotegrin (*OPG*)

Gene symbol	SNP ID	dbSNP rs	dbSNP ss	Chromosome location in NCBI build 35v1	Substitution major > minor	Location	Function	Minor allele frequency	
								In 24 AgPs	In 24 controls
RANK	RK1	7238731		18 : 58143527	G > A	5′-flank		8/48	6/48
	RK2	1805033		18 : 58143557	T > C	Exon 1	5′-UTR	8/48	6/48
	RK3	Novel	46566468	18 : 58143571	C > G	Exon 1	A2A	1/48	2/48
	RK4	Novel	46566469	18 : 58143717	G > A	Intron 1		10/48	6/48
	RK5	Novel	46566470	18 : 58143743	A > T	Intron 1		1/48	5/48
	RK6	3826618		18 : 58166522	T > C	Intron 2		11/48	10/48
	RK7	3826619		18 : 58166730	G > A	Intron 2		19/48	14/48
	RK8	3826620		18 : 58172484	T > G	Intron 3		27/48	22/48
	RK9	3810024		18 : 58176355	C > T	Intron 4		9/48	10/48
	RK10	6567270		18 : 58177985	T > A	Intron 5		22/48	19/48
	RK11	17069876		18 : 58178058	G > A	Intron 5		9/48	9/48
	RK12	6567271		18 : 58178129	T > A	Intron 5		31/48	30/48
	RK13	6567272		18 : 58178151	C > T	Intron 5		31/48	30/48
	RK14	1805034		18 : 58178221	T > C	Exon 6	A192V	31/48	30/48
	RK15	9653064		18 : 58178341	G > A	Intron 6		31/48	30/48
	RK16	12458117		18 : 58178428	G > A	Intron 6		14/48	12/48
	RK17	Novel	46566471	18 : 58179566	A > G	Intron 6		9/48	11/48
	RK18	8083511		18 : 58179635	A > C	Intron 6		23/48	20/48
	RK19	8099222		18 : 58179742	G > A	Intron 6		8/48	7/48
	RK20	Novel	46566472	18 : 58179801	G > T	Intron 6		15/48	14/48
	RK21	17069895		18 : 58179838	C > T	Intron 6		9/48	9/48
	RK22	Novel	46566473	18 : 58179873	C > T	Intron 6		1/48	0/48
	RK23	Novel	46566474	18 : 58180102	A > G	Intron 7		1/48	1/48
	RK24	Novel	46566475	18 : 58180197	T > C	Intron 7		32/48	31/48
	RK25	7239667		18 : 58180218	G > C	Intron 7		23/48	21/48
	RK26	Novel	46566476	18 : 58187704	G > A	Intron 9		1/48	0/48
	RK27	Novel	46566477	18 : 58203040	G > C	Exon 10	V548V	1/48	1/48
RANKL	RL1	2296533		13 : 42046565	T > C	Exon 1	P42P	16/48	22/48
	RL2	2277438		13 : 42053168	A > G	Intron 1		29/48	34/48
	RL3	2277439		13 : 42053443	G > A	Intron 2		29/48	34/48
	RL4	3742258		13 : 42078576	A > C	Intron 4		3/48	2/48
	RL5	9562415		13 : 42079024	T > C	Exon 5	F308F	3/48	2/48
	RL6	9567000		13 : 42079437	C > T	Exon 5	3′-UTR	3/48	2/48
	RL7	1054016		13 : 42080002	G > T	Exon 5	3′-UTR	14/48	20/48
OPG	P1	2073617		8 : 120033464	A > G	Exon 1	5′-UTR	32/48	23/48
	P2	2073618		8 : 120033233	C > G	Exon 1	N3K	31/48	37/48
	P3	3102734		8 : 120033197	G > A	Intron 1		5/48	8/48
	P4	10505346		8 : 120033024	G > T	Intron 1		7/48	7/48
	P5	11573906		8 : 120014441	C > T	Exon 2	V104M	0/48	1/48
	P6	4876869		8 : 120010458	A > G	Intron 2		11/48	16/48
	P7	3134046		8 : 120010354	A > G	Intron 2		43/48	41/48

Flank, flanking region; UTR, untranslated region; dbSNP rs. reference SNP ID; dbSNP ss, NC BI assay ID.

35 years of age and were not age-matched with the AgP patients. This selection avoided the future onset of AgP in the controls.

LD has been used to map the involvement of multiple candidate genes in complex diseases and to refine the location of disease genes in regions identified by linkage analysis (39). Estimation of the strength of pairwise LD allows the detection of risk variants by analyzing associated markers in LD. The r^2 value was used in LD analyses because the power used to indirectly detect a risk variant in n samples was equivalent to the power to directly detect it in nr^2 samples (40). Figure 2 indicates the actual value of disequilibrium coefficient pairwise r^2 and lD'l that show the pairwise relationship between two SNP positions. We detected several SNP pairs in the *RANK/RANKL/OPG* loci in high LD, but as none of the SNPs from the three genes showed evidence of associations, we could not provide a more detailed explanation of the result.

Among the SNPs in *RANK* evaluated in this study, a nonsynonymous mutation was identified in RK14 of exon 6 and interpreted as the A192V polymorphism that occurred frequently in both AgP patients and controls. Valine was detected more frequently than alanine, and this mutation is believed to encode a predicted cysteine-rich pseudorepeat in the extracellular region of RANK (37). Our data are in accordance with the *RANK* mutation in PDB (36) and in FEO (37). We found no significant association of the A192V variation in exon 6 in our set of AgP patients and the controls. Two other SNPs (RK3 and RK27) identified in the coding

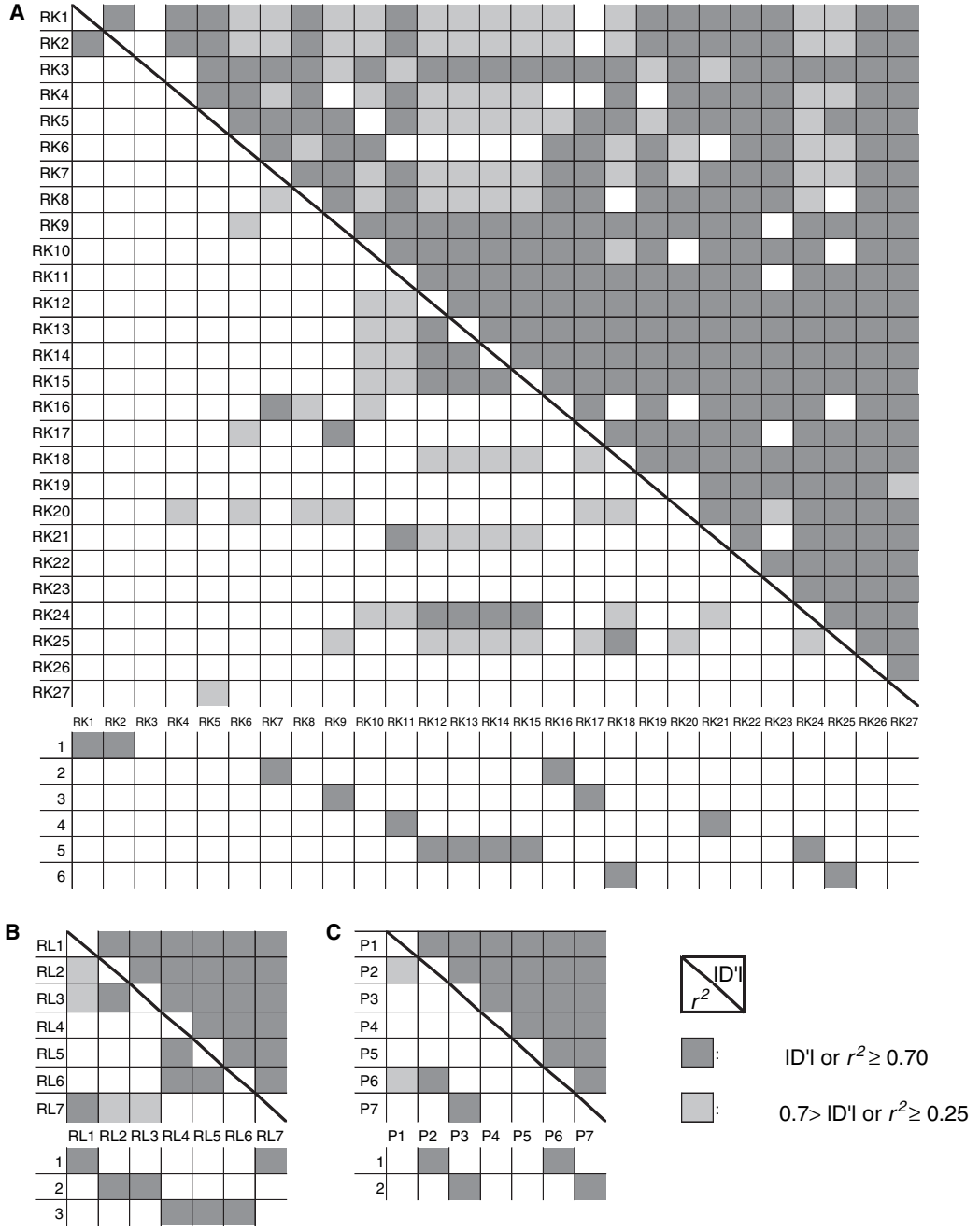

Fig. 2. Pairwise linkage disequilibrium (LD) in receptor activator of nuclear factor-κB (*RANK*) (RK), RANK ligand (*RANKL*) (RL) and osteoprotegrin (*OPG*) (P), evaluated by lD'l and r^2 estimation. Pairwise LD was determined from 48 Japanese subjects (24 patients and 24 controls). SNP pairs in high LD (lD'l or $r^2 \geq 0.70$) are represented as dark grey boxes, moderate LD ($0.7 >$ lD'l or $r^2 > 0.25$) as light grey boxes, while the remainder, in white boxes, are in low LD (lD'l or $r^2 < 0.25$). lD'l values are shown in the upper right triangle; r^2 values are in the lower left triangle. SNP IDs are shown in Table 3.

region were silent mutations and resulted in no significant difference. However, RK27, which is a novel SNP, showed a weak difference of frequency between AgP patients and controls ($p = 0.0705$) which may aid in detecting a future effect when a larger sample size is used. This result shows that *RANK* polymorphisms have a minor gene effect in the pathogenesis of AgP.

The polymorphism in exon 1 (P2) of *OPG* causes an amino acid change from lysine to aspargine. Aspargine is an uncharged polar amino acid, while lysine is a charged polar amino acid. The fact that the two amino acids belong to different groups might lead to a difference in the characteristics of the peptide. Langdahl *et al.* also reported the polymorphism in a study on osteoporotic patients and normal controls (41). A valine-to-methionine substitution at amino acid 104 (P5) in

Table 4. Association analysis of receptor activator of nuclear factor-κB (*RANK*), RANK ligand (*RANKL*) and osteoprotegrin (*OPG*) genes

	Aggressive periodontitis (AgP)				Control				Minor allele frequency		
SNP ID	Major homo	Hetero	Minor homo	Allele count	Major homo	Hetero	Minor homo	Allele count	AgP	Control	Fisher's $p < 0.05$
RK2	78	17	4	198	60	26	3	178	0.126	0.18	0.153
RK3	90	9	0	198	82	7	0	178	0.045	0.039	0.804
RK4	66	29	4	198	66	18	5	178	0.187	0.157	0.496
RK6	65	29	5	198	57	28	4	178	0.197	0.202	0.898
RK8	25	47	27	198	17	43	29	178	0.51	0.567	0.300
RK9	70	27	2	198	59	25	5	178	0.157	0.197	0.343
RK10	26	48	25	198	23	43	23	178	0.495	0.5	1
RK11	68	29	2	198	64	25	0	178	0.167	0.14	0.568
RK14	42	46	11	198	7	39	43	178	0.657	0.702	0.377
RK16	48	38	13	198	34	44	11	178	0.323	0.371	0.385
RK18	26	47	26	198	30	44	15	178	0.5	0.416	0.120
RK19	67	32	0	198	67	21	1	178	0.162	0.129	0.385
RK20	43	49	7	198	40	40	9	178	0.318	0.326	0.912
RK26	97	2	0	198	88	1	0	178	0.01	0.006	1
RK27	94	3	2	198	88	1	0	178	0.035	0.006	0.0705
RL1	35	45	19	198	27	44	18	178	0.419	0.449	0.603
RL2	40	40	19	198	9	39	41	178	0.606	0.680	0.162
RL4	86	13	0	198	75	14	0	178	0.066	0.079	0.691
P1	39	44	16	198	23	36	30	178	0.616	0.539	0.144
P2	58	38	3	198	8	37	44	178	0.778	0.702	0.0998
P3	70	26	3	198	67	18	4	178	0.162	0.146	0.775
P4	77	21	1	198	69	20	0	178	0.116	0.112	1
P5	99	0	0	198	88	1	0	178	0	0.006	0.473

AgP, aggressive periodontitis; Hetero, heterozygote; Major homo, major homozygote; Minor homo, minor homozygote; SNP, single nucleotide polymorphism.

OPG is not a novel polymorphism, but the amino acid substitution has never been reported in any bone-related diseases. As we identified this SNP in only one control subject, we excluded the possible role of this mutation in AgP. However, according to the structural domain of the *OPG* gene in humans, this mutation is located in exon 2, which contains the first two cysteine-rich motifs and 73% of the third motif from the entire four cysteine-rich motifs, all of which are responsible for the inhibition of osteoclast formation (42–44). As this mutation has not yet been reported in AgP or in other bone-related diseases, a further functional study is required.

The granulation tissues and inflammatory cells adjacent to alveolar bone loss in periodontitis expressed the elevated RANKL levels (34,35); hence, RANKL may play a crucial role in the formation and activation of osteoclasts. This suggests that the genetic polymorphisim in *RANKL* provides a possible marker for regulating bone destruction in AgP. However, in our genetic study on *RANKL*, we were unable to detect any genetic markers of *RANKL* that might be associated with AgP, although all SNPs identified are in tight LD with each other in the three groups. To our knowledge, no report has thus far described *RANKL* polymorphisms, either in AgP or other bone-related diseases.

Of all the SNPs identified in the study, SNPs in introns might affect splicing and/or transcription, and missense SNPs in exons might affect function of its product. As evaluation of the function of SNPs, based only on the nucleotide sequence is still very difficult, further functional analysis of these SNPs is needed to elucidate the molecular mechanism of aggressive periodontitis.

Although RANK/RANKL/OPG affect bone remodelling and play a vital role in both the physiological and pathological regulation of the bone, in our study they did not provide strong evidence of the major role of polymorphisms in the pathogenesis of AgP. However, the precise underlying mechanisms of bone destruction in AgP remain unknown; therefore, further research is required to evaluate other osteoclastogenesis-related cytokines in the RANK/RANKL/OPG pathway that may be the genetic markers of AgP.

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