Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 345–353

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01349.x

Altered gene expression in leukocyte transendothelial migration and cell communication pathways in periodontitis-affected gingival tissues

D. Abe, T. Kubota, T. Morozumi, T. Shimizu, N. Nakasone, M. Itagaki, H. Yoshie
Division of Periodontology, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Abe D, Kubota T, Morozumi T, Shimizu T, Nakasone N, Itagaki M, Yoshie H. Altered gene expression in leukocyte transendothelial migration and cell communication pathways in periodontitis-affected gingival tissues. J Periodont Res 2011; 46: 345–353. © 2011 John Wiley & Sons A/S

Background and Objective: Gene expression is related to the pathogenesis of periodontitis and plays a crucial role in local tissue destruction and disease susceptibility. The aims of the present study were to identify the expression of specific genes and biological pathways in periodontitis-affected gingival tissue using microarray and quantitative real-time RT-PCR analyses.

Material and Methods: Healthy and periodontitis-affected gingival tissues were taken from three patients with severe chronic periodontitis. Total RNAs from six gingival tissue samples were used for microarray analyses. Data-mining analyses, such as comparisons, gene ontology and pathway analyses, were performed and biological pathways with a significant role in periodontitis were identified. In addition, quantitative real-time RT-PCR analysis was performed on samples obtained from 14 patients with chronic periodontitis and from 14 healthy individuals in order to confirm the results of the pathway analysis.

Results: Comparison analyses found 15 up-regulated and 13 down-regulated genes (all of which showed a change of more than twofold in expression levels) in periodontitis-affected gingival tissues. Pathway analysis identified 15 up-regulated biological pathways, including leukocyte transendothelial migration, and five down-regulated pathways, including cell communication. Quantitative real-time RT-PCR verified that five genes in the leukocyte transendothelial migration pathway were significantly up-regulated, and four genes in the cell communication pathway were significantly down-regulated, which was consistent with pathway analysis.

Conclusion: We identified up-regulated genes (*ITGB-2*, *MMP-2*, *CXCL-12*, *CXCR-4* and *Rac-2*) and down-regulated genes (connexin, *DSG-1*, *DSC-1* and nestin) in periodontitis-affected gingival tissues; these genes may be related to the stimulation of leukocyte transendothelial migration and to the the impairment of cell-to-cell communication in periodontitis.

Takehiko Kubota, DDS, PhD, Division of Periodontology, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Chuo-ku, Niigata 951-8514, Japan
Tel: (+81) 25 227 2871
Fax: (+81) 25 227 0808
e-mail: kubota@dent.niigata-u.ac.jp

Key words: DNA microarray; gene expression; periodontitis; pathogenesis of periodontal diseases

Accepted for publication December 15, 2010

Periodontal diseases are caused by infection with periodontopathic bacteria, and host cells exhibit an immune response to this infection, which results in periodontal tissue destruction (1,2). Destruction of periodontal soft and hard tissue is the result of bursts of host-parasite balance occurring for short periods of time at individual, random sites throughout the mouth that are affected by periodontitis; a higher frequency of this activity may take place during certain periods of an individual's life, when collagenolysis may also be found (3,4). Periodontal disease progression is also related to host-based risk factors, such as genetics, age, gender and systemic conditions (5). We have previously investigated the relationship between gene polymorphisms and periodontitis, focusing on the effects of single nucleotide polymorphisms of interleukin (IL)-1 and the IL-1 receptor antagonist (6), of tumor necrosis factor receptor type 2 (7), of vitamin D (8) or Fc receptors (9), and of MMPs (10). To clarify the pathogenesis of periodontitis, gene polymorphism and *in situ* gene-expression analyses were thus considered necessary.

Several studies have reported that periodontal tissue remodeling and destruction are tightly regulated by MMPs and their specific inhibitors, known as TIMPs (11,12). It has been reported that both MMP levels and MMP/TIMP ratios (13,14), as well as the levels of inflammatory cytokines, such as IL-1 beta, tumor necrosis factor, IL-12 and IL-17, and the levels of anti-inflammatory cytokine such as IL-10, are elevated in periodontitis-affected gingival tissues (15–18). These reports suggest that a number of genes involved in biological, immunological and metabolic pathways could be simultaneously regulated by host-protective mechanisms in periodontitis-affected gingival tissues.

Periodontal diseases are caused by the expression of multiple genes involved in complex pathways related to disease susceptibility. Therefore, it is desirable to clarify the pathogenesis of periodontitis using a comprehensive approach to identify simultaneously up-regulated or down-regulated genes in gingival tissues at sites of active periodontal tissue destruction.

Microarray analyses have recently been applied to analyze comprehensive gene-expression profiles in various diseases, such as cancer (19), rheumatology disorders (20), Alzheimer's disease (21) and periodontitis (22). Demmer *et al*. (23) compared periodontitis-affected gingival sites with healthy sites using microarrays and identified differentially expressed pathways, such as apoptosis, the antimicrobial humoral response and angiogenesis. Another report examined gingival tissues from refractory periodontitis patients and identified a set of candidate genes, including MMP-1 and MMP-3, as diagnostic and screening markers for high-risk individuals (24). Moreover, the induction and resolution phases of gingival inflammation were recently analyzed using whole-transcriptome gene-expression analysis of gingival tissues biopsied at different time-points (25). These reports suggest that microarray analyses may provide an insight into the associations between gene expression and clinical signs. However, the etiology of periodontitis is complex, and no reports on quantitative gene-expression analyses for functional biological pathways in periodontitis are currently available.

The purpose of the present study was to identify specific gene-expression and biological relationships in periodontitis-affected gingival tissue using microarrays and quantitative real-time RT-PCR.

Material and methods

Subjects

This study was approved by the regional ethics committee of the Faculty of Dentistry, Niigata University, and all subjects provided written informed consent before participation. A total of 28 subjects were recruited from patients attending Niigata University Medical & Dental Hospital, Niigata, Japan, between November 2007 and May 2008. All subjects were systemically healthy Japanese people who possessed a minimum of 20 teeth and had good oral hygiene, did not have diabetes, were not pregnant, were not current smokers and had taken no systemic antibiotics or anti-inflammatory drugs within the previous 6 mo. Women accounted for 46.4% of the subjects. Fourteen patients with generalized severe chronic periodontitis and who had received conventional periodontal treatment more than 4–8 wk before the study were selected (Group P). Another 14 individuals with good clinical periodontal health and no history of periodontal disease, impacted teeth or severe dental caries were also enrolled (Group H). The clinical and demographic characteristics of the subjects are shown in Table 1. The characteristics were comparable in each group.

Collection of gingival tissue samples

Two distinct gingival samples, including healthy and periodontitis-affected gingiva, were taken from three patients (Microarray-case 1, Microarray-case 2 and Microarray-case 3) in Group P and were used for microarray analysis. Only periodontitis-affected gingival samples were taken from the other 11 patients. Fourteen healthy gingival tissue samples were harvested from Group H subjects. Diseased sites showed bleeding on probing and a gingival index of ≥ 1, and had a probing pocket depth and clinical attachment loss of ≥ 5 mm. Healthy sites had a probing pocket depth of ≤ 2 mm with neither clinical attachment loss nor gingival inflammation. Periodontitis-affected and healthy gingival (connective and epithelial) tissue samples were respectively obtained during periodontal flap surgery and tooth extraction, as described previously (5).

RNA extraction

Immediately after sampling, gingival tissues were immersed in 500 μL of RNA stabilization reagent (RNA later; Qiagen, Valencia, CA, USA), removed and kept overnight at 4°C. Samples were homogenized thoroughly using a tissue homogenizer (Polytron homogenizer; Kinematica, Lucerne, Switzerland) and total RNA was isolated using

Table 1. General characteristics and clinical periodontal variables of study participants

	Microarray-case 1		Microarray-case 2		Microarray-case 3		Group P (n = 14) Mean ± SD	Group H (n = 14)
Age (years)	59		64		70		58.0 ± 16.0	35.0 ± 17.1
Gender	Male		Female		Male		Male:7/female:7	Male:8/female:6
Number of teeth	25		26		24		20.0 ± 2.0	30.0 ± 2.0
	P	H	P	H	P	H		
Sampling sites	32 db	42 mb	47 db	47 mb	26 dp	25 mp		
PPD (mm)	6	3	6	2	6	3	6.5 ± 2.0	1.6 ± 0.2
CAL (mm)	8	2	7	2	7	2	7.0 ± 1.5	1.5 ± 0.4
GI	1	0	1	0	1	0	1.2 ± 0.5	0.0 ± 0.0
BOP	+	−	+	−	+	−	+	−

+: presence of BOP; −: absence of BOP; b, buccal; BOP, bleeding on probing; CAL, clinical attachment level; d, distal; GI, gingival index; H, healthy site; m, mesial; P, periodontitis site; p, palatal; PPD, probing pocket depth.
Group H: 14 individuals with good clinical periodontal health and no history of periodontal disease, impacted teeth or severe dental caries.
Group P: 14 patients with generalized severe chronic periodontitis who had received conventional periodontal treatment more than 4–8 wk before the study.

the RNAiso® reagent (TaKaRa Bio Inc., Otsu, Japan). Quality control and quantification of total RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) before microarray experiments. Two micrograms of extracted total RNA from Microarray-case1 and 3 μg of extracted total RNA from each Microarray-case2 and 3 were used for quality control analysis. Peaks corresponding to 18S and 28S RNA showed that total RNA from the gingival tissue was of sufficiently high quality for microarray analyses.

Reverse transcription, *in vitro* complementary RNA synthesis and microarray hybridization

Three micrograms of total RNA was reverse-transcribed using a GeneChip® T7-Oligo(dt) Promoter Primer kit (Affymetrix, Santa Clara, CA, USA) and a complementary DNA Synthesis kit (M-MLV version; TaKaRa Bio). Synthesis of biotinylated complementary RNA was performed using a GeneChip® IVT Labeling kit (Affymetrix) for *in vitro* transcription. After fragmentation, 10 μg of complementary RNA was hybridized at 45°C for 16 h onto a GeneChip® Human Genome U133 Plus 2.0 Array® (Affymetrix). GeneChips were washed and stained in a GeneChip® Fluidics Station 450 (Affymetrix). Fluorescence intensities for chips were examined on a GeneChip Scanner 3000 7G (Affymetrix). Gene-expression profiles in periodontal-affected gingiva were compared with those of clinically healthy sites.

Microarray data analyses

Single array analysis and comparison analysis were performed using Microarray Suite version 5.0 (MAS5.0), with Affymetrix default settings and global scaling, as a normalization method for the complete database comprising six measurements of expression (three in periodontitis and three in a healthy state) of 38,500 genes.

The trimmed mean target intensity for each array was arbitrarily set at 500. Data were filtered to ensure both statistical and biological significance. Values were normalized against the median signal values for each array. Genes that showed a statistically significant higher level of expression when compared with the control group were selected using a paired t-test ($p < 0.05$). Healthy and periodontitis sites were compared, and genes found to exhibit at least twofold changes in their expression levels between periodontitis and control sites were selected (26). Data sets presented in the study have been deposited in the Gene expression Omnibus (GEO, National Center for Biotechnology Information) database (accession no.: GSE 23586).

Gene Ontology analysis

The Gene Ontology (GO) database contains standardized biological terms used to annotate gene products. In total, it comprises around 16,000 terms, divided into three categories: molecular function; biological processes; and cellular component (27). Briefly, molecular function refers to what a gene product does at the biological level; biological process refers to a broad biological objective; and cellular component refers to the location of a gene product within cellular structures or within macromolecular complexes (28). The procedure described by Zheng *et al.* (29) was followed in order to perform GO analysis. Gene sets differentially expressed under experimental conditions were fed into the NetAffx Analysis Center in the Expression Omnibus Database (Affymetrix) and p-values were computed for each GO term using Fisher's exact test. The categories that were particularly enriched or depleted in a set of significantly differentiated genes were extracted as described previously (30).

Kyoto Encyclopedia of Gene and Genomes pathway analysis

Functional assignment in the Kyoto Encyclopedia of Gene and Genomes pathway (KEGG) is the process of

linking a set of genes in the genome with a network of interacting molecules in the cell, such as a pathway or complex representing a higher-order biological function (31). To analyze the biological profiles included in each group, KEGG pathway analyses were performed as described previously (32).

Post-hoc confirmation using quantitative real-time RT-PCR

Quantitative real-time RT-PCR primers and probes for integrin beta 2 (*ITGB2*; Hs00164957_ml), *MMP-2* (Hs01548724_ml), chemokine ligand 12 (*CXCL-12*; Hs00171022_ml), chemokine receptor 4 (*CXCR-4*; Hs00607978_ml), Ras-related C3 botulinum toxin substrate 2 (*Rac2*; Hs00427439_gl), connexin (Hs01591801_sl), desmogrein 1 (*DSG-1*; Hs00355084_ml), desmocollin 1 (*DSC-1*; Hs00245189_ml) and nestin (Hs00707120_sl) were purchased from Applied Biosystems (Foster City, CA, USA). Quantitative real-time RT-PCR to confirm the expression of genes belonging to the pathways was performed using a sequence detection system (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems). Briefly, a reaction solution composed of premixed PCR buffer (TaqMan Universal PCR Master Mix; Applied Biosystems), forward and reverse primers (final concentration, 300 nM each), a specific probe (TaqMan probe; Applied Biosystems; final concentration, 200 nM) and a complementary DNA (cDNA) mixture (25 ng) were mixed in a total volume of 25 μL. Conditions for quantitative real-time RT-PCR were as follows: preheating at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of shuttle heating at 95°C for 15 s and at 60°C for 1 min. All PCR amplifications were run in duplicate. Sequence detection software (ABI Prism SDS version 2.0; Applied Biosystems) was used to analyze the standards and to carry out quantification. The relative quantity of each mRNA was normalized against the relative quantity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) determined using specific endogenous control primers (Predeveloped TaqMan Assay Reagent Endogenous Control; Applied Biosystems). The comparative threshold cycle (Ct) (i.e. the number of PCR cycles necessary to obtain the threshold signal of fluorescence) method was used to

Table 2. Commonly up-regulated genes

Gene	Microarray-case 1	Microarray-case 2	Microarray-case 3	Mean[a]
Membrane-spanning 4-domains, subfamily A, member 1	4.5	2.3	2.8	3.2
Immunoglobulin J polypeptide, linker protein	2.5	2.1	3.3	2.6
Chemokine (C-X-C motif) ligand 12	3.1	1.3	3.4	2.6
SAM domain, SH3 domain and nuclear localization signals 1	3.1	1.6	2.9	2.5
Chemokine (C-X-C motif) ligand 1	2.6	2.0	2.9	2.5
Complement factor H	2.2	2.3	2.6	2.4
Scavenger receptor class A, member 5	2.4	1.8	2.8	2.3
Osteoglycin	2.8	1.2	2.6	2.2
Matrix Gla protein	1.9	2.4	2.2	2.2
Interleukin 1beta	3.3	1.7	1.4	2.1
Platelet-derived growth factor D	1.9	2.0	2.3	2.1
Lymphocyte cytosolic protein 2	2.6	1.5	2.0	2.0
EPH receptor A3	2.4	1.7	2.0	2.0
Osteomodulin	2.4	1.1	2.4	2.0
Fibronectin 1	2.0	1.8	2.1	2.0

[a]Changes of more than twofold are presented.

Table 3. Commonly down-regulated genes

Gene	Microarray-case 1	Microarray-case 2	Microarray-case 3	Mean[a]
Premature ovarian failure, 1B	−3.8	−2.2	−5.9	−4.0
Keratin 17	−2.0	−3.8	−3.9	−3.2
Keratin 10	−3.9	−1.9	−3.7	−3.2
Dermokine	−2.7	−2.1	−4.6	−3.1
WAP four-disulfide core domain 5	−2.4	−3.1	−3.9	−3.1
Peptidase inhibitor 3, skin-derived	−1.7	−2.2	−5.2	−3.0
Erythrocyte membrane protein band 4.1 like 4B	−2.8	−1.4	−4.3	−2.8
Epiplakin 1	−1.8	−2.3	−4.0	−2.7
CDNA clone IMAGE:4733238	−2.8	−1.3	−3.9	−2.7
ATPase, class V, type 10B	−2.7	−1.5	−3.4	−2.5
Hypothetical protein LOC283666	−1.7	−2.0	−3.4	−2.4
CD36 molecule	−1.4	−1.9	−3.6	−2.3
RAR-related orphan receptor A	−1.5	−2.0	−3.1	−2.2

[a]Changes of more than twofold are presented.

Table 4. Differentially expressed pathways, determined by pathway analysis

Pathways	*p*-value	Counts
Up-regulated pathways		
Cytokine-cytokine receptor interaction	0.0001	14
Complement and coagulation cascades	0.0006	6
Hematopoietic cell lineage	0.0009	4
Leukocyte transendothelial migration	**0.0017**	**6**
Bladder cancer	0.0035	4
Alzheimer's disease	0.0084	3
ECM-receptor interaction	0.0098	5
Gap junction	0.0124	3
Primary immunodeficiency	0.0155	3
Sulfur metabolism	0.0184	2
Toll-like receptor signaling pathway	0.0186	5
Regulation of actin cytoskeleton	0.0222	4
Natural killer cell mediated cytotoxicity	0.0288	3
Wnt signaling pathway	0.0386	3
Pathogenic *Escherichia coli* infection – EPEC	0.0415	3
Down-regulated pathways		
Cell communication	**0.0001**	**4**
Amyotrophic lateral sclerosis	0.0061	2
Arachidonic acid metabolism	0.0188	2
Arginine and proline metabolism	0.0201	2
Tetrachloroethene degradation	0.0434	1

Counts, gene counts included in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.
p-value, significant at $p < 0.05$, as determined by pathway enrichment analysis.
Bold, pathways selected for quantitative real-time RT-PCR analyses.

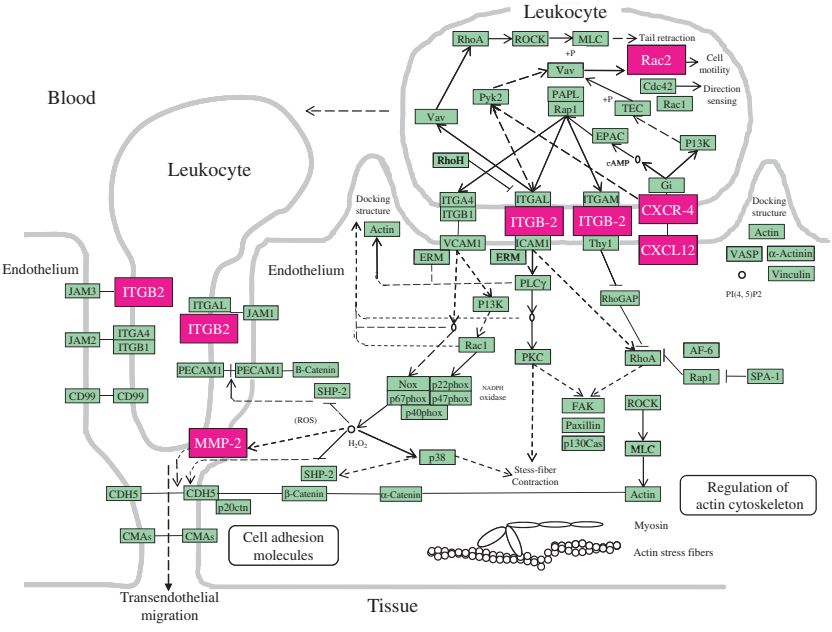

Fig. 1. Kyoto Encyclopedia of Gene and Genomes (KEGG) map of the leukocyte transendothelial migration pathway. Genes shown with pink backgrounds are expressed at higher levels in periodontitis-affected gingival tissues than in healthy tissues, as determined by pathway analysis. CXCL-12, chemokine ligand 12; CXCR-4, chemokine receptor 4; ITGB-2, integrin beta 2; MMP-2, matrix metalloproteinase 2; Rac2, Ras-related C3 botulinum toxin substrate 2.

quantify the amplified transcripts. Differences in transcript levels between healthy and periodontitis patients were analyzed using the Mann–Whitney *U*-test ($p < 0.05$).

Results

Microarray analyses

The expression of 41–955 genes was significantly elevated in periodontitis tissues, and the expression of 32–402 genes was significantly decreased in the three chronic periodontitis patients. We identified 15 commonly up-regulated genes (Table 2) and 13 commonly down-regulated genes (Table 3) in periodontitis-affected gingival tissues, showing at least a twofold change in expression. Inflammatory cytokines, such as IL-1beta, chemokine ligands and their receptors, which are associated with immunological responses against inflammation, were detected as up-regulated genes. In contrast, genes for cytoskeletal or epithelial components, including keratins, epiplakins and dermokines, were found to be down-regulated.

Gene ontological analysis found 45 significantly and differentially up-regulated ontological groups in periodontitis-affected gingival tissues, including inflammatory responses, immune-system processes, chemotaxis, taxis, response to injury, leukocyte migration, cell localization, cell motility and cell migration (Supplementary Table S1). In contrast, 24 groups, including epidermal development, ectoderm development, keratinocyte differentiation, organ development, keratinization, epidermal cell differentiation and multicellular organismal development, were found to be down-regulated (Supplementary Table S2). The complete list of gene ontology groups differentially expressed between diseased and healthy gingival tissue at $p < 0.05$ can be viewed in Tables S1 and S2.

Pathway analysis identified significantly and differentially expressed biological pathways in periodontitis-affected gingival tissues. As shown in Table 4, 15 pathways, including leukocyte transendothelial migration, cytokine-to-cytokine receptor interac-

tion, complement and coagulation cascades, hematopoietic cell lineage, Alzheimer's disease, Wnt-signaling and toll-like receptor signaling pathways, were significantly up-regulated, showing at least twofold changes in expression. In contrast, there were five significantly down-regulated pathways, including the cell communication and arachidonic acid metabolism pathways. We decided to focus our attention on the leukocyte transendothelial migration pathway (Fig. 1) and the cell communication pathway (Fig. 2), because of their statistical significance ($p < 0.05$), their known functional relevance and their location in periodontal tissues.

Quantitative real-time RT-PCR

In order to validate the pathway analysis, five genes in the leukocyte transendothelial migration pathway (*ITGB-2*, *MMP-2*, *CXCL-12*, *CXCR-4* and *Rac-2*), and four genes in the cell communication pathway (connexin, *DSG-1*, *DSC-1* and nestin), which showed a high change rate in each pathway, were selected and analyzed by quantitative real-time RT-PCR. All five genes of the leukocyte transendothelial migration pathway that were examined were significantly up-regulated (Fig. 3), while all four genes belonging to the cell communication pathway were significantly down-regulated (Fig. 4). These results were consistent with those of pathway analysis.

Discussion

The present study successfully described the comprehensive gene-expression profiles of gingival tissues using microarray analyses, including comparisons, gene ontology and pathway analysis. In addition, quantitative real-time RT-PCR was performed in order to validate microarray data. We identified several up-regulated (*ITGB-2*, *MMP-2*, *CXCL-12*, *CXCR-4* and *Rac-2*) and down-regulated (connexin, *DSG-1*, *DSC-1* and nestin) gene-expression profiles for the first time in periodontitis-affected gingival tissues, compared with healthy controls.

There was a wide range of up- or down-regulated genes among the microarray data obtained in this study. This may be a result of individual differences between subjects (e.g. degree of inflammation and gingival tissue quality). However, statistical analyses identified several common differentially expressed genes in periodontitis-affected gingival tissues. Diseased and control samples were taken from separate parts of each gingival tissue in order to standardize the individual gene-expression background, and 15 common up-regulated genes, such as IL-1beta and chemokines, were found to be involved in inflammatory and immunological responses. In contrast, the 13 down-regulated genes included factors for epithelial constructive and cytoskeletal proteins, such as keratins. These gene-expression profiles indicate that genes involved in chronic inflammatory stimuli and immune responses are important in the gingival tissue destruction of periodontitis. In the

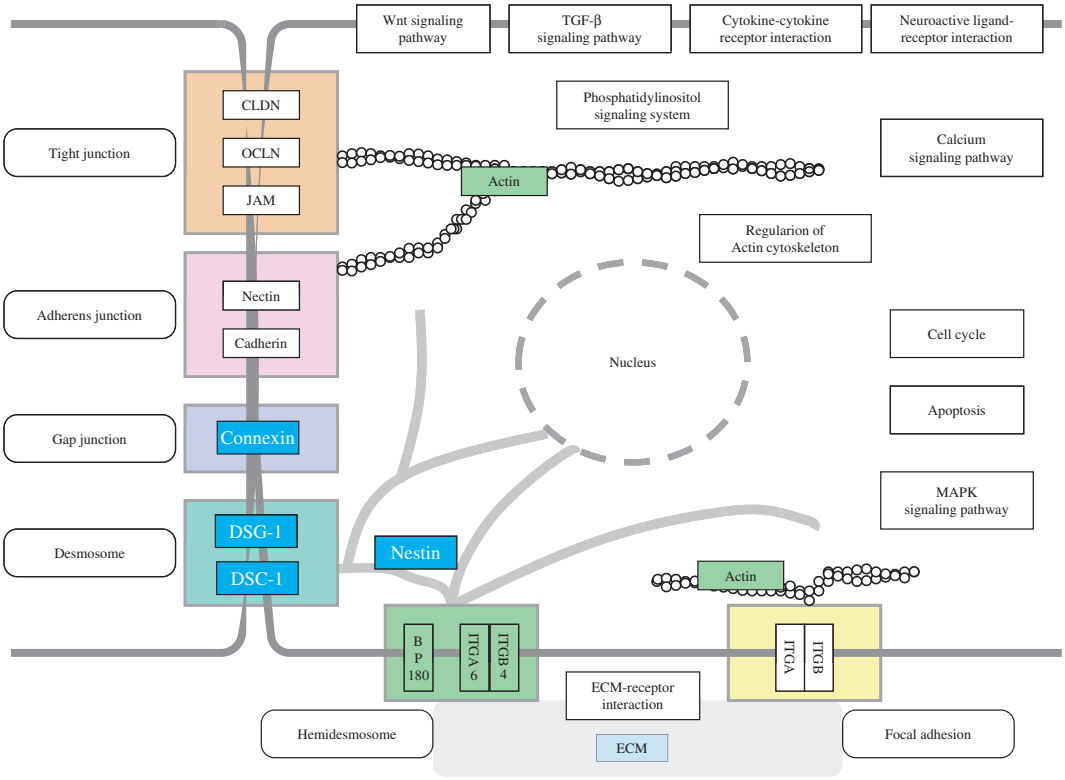

Fig. 2. Kyoto Encyclopedia of Gene and Genomes (KEGG) map of the cell communication pathway. Genes shown with sky blue backgrounds are expressed at lower levels in periodontitis-affected gingival tissues than in healthy tissues, as determined by pathway analysis. DSC-1, desmocollin 1; DSG-1, desmogrein 1.

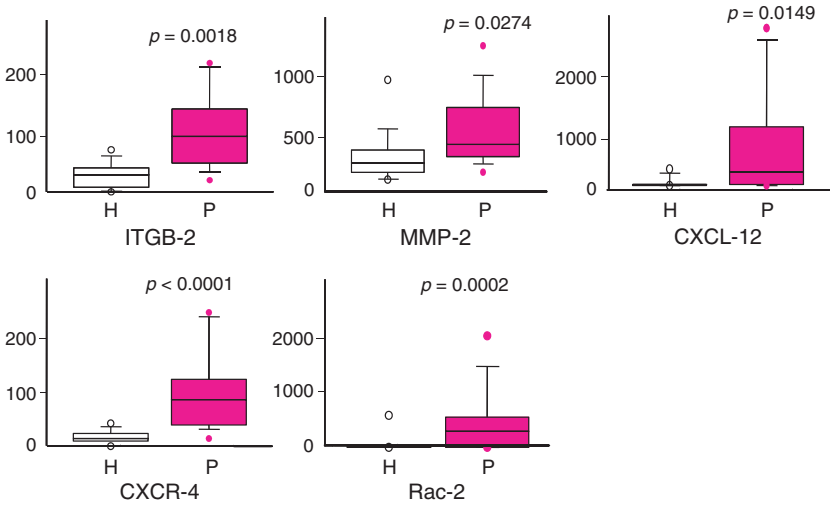

Fig. 3. Gene transcript levels, relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level, for integrin beta 2 (ITGB-2), matrix metalloproteinase-2 (MMP-2), chemokine ligand 12 (CXCL-12), chemokine receptor 4 (CXCR-4) and ras-related C3 botulinum toxin substrate 2 (Rac2), which belong to the leukocyte transendothelial migration pathway. Statistical analysis was performed using the Mann–Whitney *U*-test ($p < 0.05$). *y*-axis: messenger RNA levels relative to GAPDH. H: Group H, 14 individuals with good clinical periodontal health and no history of periodontal disease, impacted teeth or severe dental caries. P: Group P, 14 patients with generalized severe chronic periodontitis who had received conventional periodontal treatment more than 4–8 wk before the study. Box-and-whisker plots: the bottoms and the tops of the boxes represent the lower and the upper quartiles, respectively. The line near the middle of the box is the median. The ends of the vertical lines, or 'whiskers', indicate the minimum and maximum data values.

study, we focused on two pathways – the leukocyte transendothelial migration pathway (which was up-regulated) and the cell communication pathway (which was down-regulated), as well as their constituent genes.

The leukocyte transendothelial migration pathway is reported to be important in various diseases, including atherosclerosis (33), rheumatic disease (34), diabetes (35) and cancers (36), in addition to periodontitis, as shown in the present study. In particular, the *ITGB-2*, *MMP-2*, *CXCL-12*, *CXCR-4* and *Rac-2* genes are included in the leukocyte transendothelial migration pathway in KEGG, and these genes showed significant up-regulation. *CXCL-12* and *MMP-2* are known to be expressed on the endothelium of blood vessels, while *CXCR-4*, *ITGB2* and *Rac-2* are expressed in leukocytes (http://www.genome.jp/kegg/pathway/hsa/hsa04670.html).

The leukocyte transendothelial migration pathway plays an important role in the process of intravascular leukocyte connection with the vascular endothelium in order to facilitate the transition of leukocytes into tissues. Therefore, up-regulated leukocyte transmigration in periodontitis-affected gingival tissue may be associated with activated immune responses that are dependent on leukocyte migration across endothelial cells. Leukocyte migration through the epithelial lining of the gingival pocket is also reported to constitute the first line of defense against plaque bacteria (37). On the other hand, the migration, through the junctional epithelium of leukocytes involved in host defence, may play a destructive role between the tooth and the junctional epithelium, leading to the progression of periodontitis.

CXCR-4 and its ligand, CXCL-12, are known to be involved in the trafficking of leukocytes into and out of extravascular tissues (38). As CXCL-12 is a powerful chemoattractant for hematopoietic cells, including neutrophils, it may facilitate transmigration across endothelial cell barriers (39). In periodontitis, CXCL-12 has been reported to act as a chemoattractant for host defense cells and therefore in gingival crevicular fluid it may serve as a biomarker for periodontal diseases (38). Thus, the locally enhanced *CXCL12* gene expression may attract these inflammatory cells *in situ*.

Rac-2, a member of the small Rho GTPase family, also plays essential roles in coordinating directional migration and superoxide production during neutrophil responses to chemoattractants (40). The up-regulated expression of the *Rac-2* gene in periodontitis-affected gingival tissue, which can be triggered by extracellular chemotactic stimuli, such as CXCL12, may synergistically up-regulate leukocyte migration.

MMP-2 (gelatinase-A, 72 kDa) is a key enzyme involved in physiological tissue remodeling and pathological extracellular matrix degradation in the pathogenesis of periodontal diseases (41). In particular, MMP-2 protein cleaves both collagenous (type IV collagen) and noncollagenous components of the extracellular matrix constitutively expressed by most connective tissue cells, including endothelial cells, osteoblasts, fibroblasts and myoblasts (42,43). Elevated MMP-2 levels in gingival tissue or GCF have been observed in inflammatory sites in periodontitis (44). These up-regulated genes may thus cooperate in the pathogenesis of periodontitis.

It is known that the destruction of periodontal tissues is initiated by loss of attachment between cell and tooth and/or cell-to-cell adhesions. These biological connections are tightly controlled by multiple genes belonging to biological pathways, such as the cell communication pathway. Connexin, *DSG-1* and *DSC-1* are important for cell-to-cell junctions (45), and these genes were found to be significantly down-regulated in periodontitis-affected gingival tissues. These results indicate that biological cell communication is down-regulated in periodontal destructive lesions.

The down-regulation of cytoskeletal genes observed in the present study could be associated with weakened cell-to-cell adhesion in periodontal tissue caused by periodontitis. Decreased

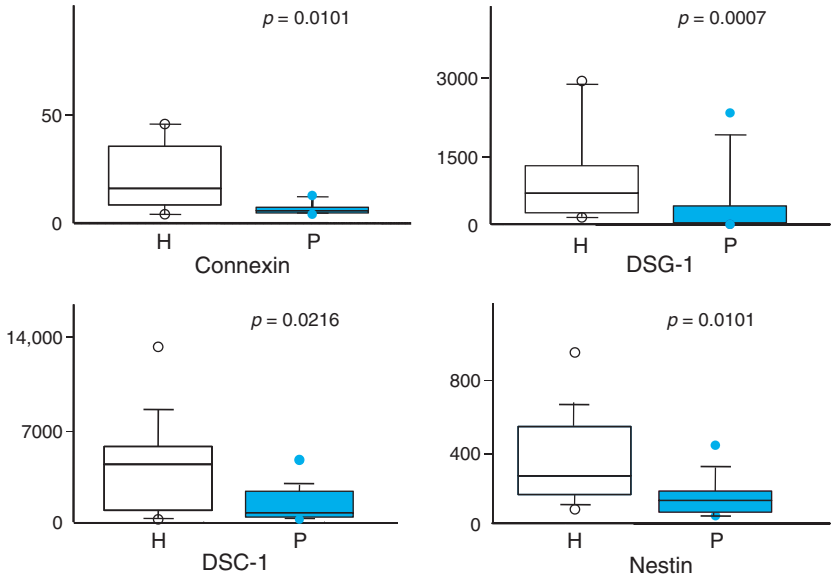

Fig. 4. Gene transcript levels, relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level, for connexin, desmogrein 1 (DSG-1), desmocollin 1 (DSC-1) and nestin, which belong to the cell communication pathway. Statistical analysis was performed using the Mann–Whitney *U*-test ($p < 0.05$). *y*-axis: messenger RNA levels relative to GAPDH. H: Group H, 14 individuals with good clinical periodontal health and no history of periodontal disease, impacted teeth or severe dental caries. P: Group P, 14 patients with generalized severe chronic periodontitis who had received conventional periodontal treatment more than 4–8 wk before the study. Box-and-whisker plots: the bottoms and the tops of the boxes represent the lower and the upper quartiles, respectively. The line near the middle of the box is the median. The ends of the vertical lines, or 'whiskers', indicate the minimum and maximum data values.

DSC-1 mRNA in gingival tissue from patients with refractory periodontitis was also reported by Kim *et al.* (24). As gap junction connexin is considered to be an important marker of tissue integrity, differentiation and communication in squamous epithelium (46), impairment of these functions may also weaken periodontal tissues. Thus, the down-regulation of connexin gene expression and decreased tissue integrity may cause destruction of periodontal tissue. Nestin is an intermediate filament closely related to developmental processes such as neurogenesis, myogenesis, and developing eyes and tooth buds (47). Thus, the expression of nestin mRNA may lead to decreased regeneration and/or repair of periodontal tissues, resulting in the progression of chronic periodontitis.

We believe, from a functional viewpoint, that these genes play an important role in the development and progression of periodontitis. This study also suggests that microarray data mining and quantitative real-time RT-PCR measurements are useful tools in detecting pathological genes in periodontitis. However, further investigation to identify functional relationships with other biological pathways is necessary.

In conclusion, we identified up-regulated genes (*ITGB-2*, *MMP-2*, *CXCL-12*, *CXCR-4* and Rac-2) and down-regulated genes (connexin, *DSG-1*, *DSC-1* and nestin) in periodontitis-affected gingival tissues that may be related to the stimulation of leukocyte transendothelial migration and the impairment of cell-to-cell communication in periodontitis.

Acknowledgements

This work was supported by Grants-in Aid for Scientific Research (no. 19390635 to T. Kubota; and no. 21592622 to H. Yoshie) from the Japan Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a 2006 Niigata University Research Project Grant (to T. Kubota). The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Significantly up-regulated 45 ontological groups in periodontitis-affected gingival tissues.

Table S2. Significantly down-regulated 24 ontological groups in periodontitis-affected gingival tissues.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal disease. *Periodontol 2000* 1994;**5:**78–111.
2. Moore WE, Moore LV. The bacteria of periodontal diseases. *Periodontol 2000* 1994;**5:**66–77.
3. Socransky SS, Haffajee AD. New concept of destructive periodontal disease. *J Clin Periodontol* 1984;**11:**21–32.
4. Van der Zee E, Everts V, Beertsen W. Cytokines modulate routes of collagen breakdown. *J Clin Periodontol* 1997;**24:** 297–305.
5. Kinane DF, Peterson M, Stathopoulou PG. Environmental and other modifying factors of the periodontal diseases. *Periodontol 2000* 2006;**40:**107–119.
6. Tai H, Endo M, Shimada Y *et al.* Association of interleukin-1 receptor antagonist gene polymorphism with early onset periodontitis in Japanese. *J Clin Periodontol* 2002;**29:**882–888.
7. Shimada Y, Tai H, Endo M, Kobayashi T, Akazawa K, Yamazaki K. Association of tumor necrosis factor receptor type 2 + 587 gene polymorphism with severe chronic periodontitis. *J Clin Periodontol* 2004;**31:**463–469.
8. Yoshihara A, Sugita N, Yamamoto K, Kobayashi T, Miyazaki H, Yoshie H. Analysis of vitamin D and Fcgamma receptor polymorphism in Japanese

patients with generalized early-onset periodontitis. *J Dent Res* 2001;**80:**2051–2054.
9. Kobayashi T, Ito S, Kuroda T *et al*. The interleukin-1 and Fcgamma receptor gene polymorphisms in Japanese patients with rheumatoid arthritis and periodontitis. *J Periodontol* 2007;**78:**2311–2318.
10. Itagaki M, Kubota T, Tai H, Shimada Y, Morozumi T, Yamazaki K. Matrix metalloproteinase-1 and -3 gene promoter polymorphisms in Japanese patients with periodontitis. *J Clin Periodontol* 2004; **31:**764–769.
11. Kubota T, Itagaki M, Hoshino C *et al*. Altered gene expression levels of matrix metalloproteinases and their inhibitors in periodontitis-affected gingival tissue. *J Periodontol* 2008;**79:**166–173.
12. Kinane DF. Regulations of tissue destruction and homeostasis as diagnostic aids in periodontology. *Periodontol 2000* 2000;**24:**215–225.
13. Kubota T, Nomura T, Takahashi T, Hara K. Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis affected human gingival tissue. *Arch Oral Biol* 1996;**41:**253–262.
14. Kubota T, Matsuki Y, Nomura T, Hara K. *In situ* hybridization study on tissue inhibitors of metalloproteinases (TIMPs) mRNA-expressing cells in human inflamed gingival tissue. *J Periodontal Res* 1997;**32:**467–472.
15. Tokoro Y, Yamamoto T, Kara K. IL-1 beta mRNA as the predominant inflammatory cytokine transcript: correlation with inflammatory cell infiltration into human gingiva. *J Oral Pathol Med* 1996;**25:**225–231.
16. Roberts EA, McCaffery KA, Michalek SM. Profile of cytokine mRNA expression in chronic adult periodontitis. *J Dent Res* 1997;**76:**1833–1839.
17. Garlet GP, Cardoso CR, Campanelli AP, Martins W Jr, Silva JS. Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression? *J Periodontal Res* 2006;**41:**580–584.
18. Honda T, Aoki Y, Takahashi N *et al*. Elevated expression of IL-17 and IL-12 genes in chronic inflammatory periodontal disease. *Clin Chim Acta* 2008;**395:**137–141.
19. Chung CH, Bernard PS, Perou CM. Molecular portraits and the family tree of cancer. *Nat Genet* 2002;**32:**533–540.
20. Thornton S, Sowders D, Aronow B *et al*. DNA microarray analysis reveals novel gene expression profiles in collagen-induced arthritis. *Clin Immunol* 2002;**105:** 155–168.
21. Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG, Lukiw WJ. Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J Neurosci Res* 2002;**70:**462–473.
22. Beikler T, Peters U, Prior K, Eisenacher M, Flemmig TF. Gene expression in periodontal tissues following treatment. *BMC Med Genomics (serial online)*, 2008;**1:**30.
23. Demmer RT, Behle JH, Wolf DL *et al*. Transcriptomes in healthy and diseased gingival tissues. *J Periodontol* 2008;**79:** 2112–2124.
24. Kim DM, Ramoni MF, Nevins M, Fiorellini JP. The gene expression profile in refractory periodontitis patients. *J Periodontol* 2006;**77:**1043–1050.
25. Offenbacher S, Barros SP, Paquette DW *et al*. Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans. *J Periodontol* 2009;**80:**1963–1982.
26. Koshi R, Sugano N, Orii H, Fukuda T, Ito K. Microarray analysis of nicotine-induced changes in gene expression in a macrophage- like human cell line. *J Periodont Res* 2007;**42:**518–526.
27. Lewin A, Grieve IC. Grouping Gene Ontology terms to improve the assessment of gene set enrichment in microarray data. *BMC Bioinformatics (serial online)*, 2006;**7:**426.
28. The Gene Ontology Consortium. Creating the gene ontology resource: design and implementation. *Genome Res* 2001;**11:** 1425–1433.
29. Zheng G, George EO, Narasimhan G. Neural network classifiers and gene selection methods for microarray data on human lung adenocarcinoma. In: Shoemaker JS, Lin SM, eds. *Methods of Microarray Data Analysis IV* Berlin: Springer, 2005:207–222.
30. Zeeberg BR, Feng W, Wang G *et al*. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol* 2003;**4:**28.
31. Kanehisa M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000;**28:**27–30.
32. Handfield M, Mans JJ, Zheng G *et al*. Distinct transcriptional profiles characterize oral epithelium-microbiota interactions. *Cell Microbial* 2005;**7:**811–823.
33. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. *Atherosclerosis* 2003;**170:**191–203.
34. Szekanecz Z, Koch AE. Vascular involvement in rheumatic disease: 'vascular rheumatology'. *Arthritis Res Ther* 2008;**10:**224.
35. Jager A, van Hinsbergh VW, Kostense PJ *et al*. Increased levels of soluble vascular cell adhesion molecule 1 are associated with risk of cardiovascular mortality in type 2 diabetes: the Hoorn study. *Diabetes* 2000;**49:**485–491.
36. Miles FL, Pruitt FL, van Golen KL, Cooper CR. Stepping out of the flow: capillary extravasation in cancer metastasis. *Clin Exp Metastasis* 2008;**25:**305–324.
37. Muller WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* 2003;**24:**327–334.
38. Havens AM, Chiu E, Taba M *et al*. Stromal-derived factor-1 alpha (CXCL12) levels increase in periodontal disease. *J Periodontol* 2008;**79:**845–853.
39. Imai K, Kobayashi M, Wang J *et al*. Selective transendothelial migration of hematopoietic progenitor cells: a role in homing of progenitor cells. *Blood* 1999;**93:**149–156.
40. Zhang H, Sun C, Glogauer M, Bokoch GM. Human neutrophils coordinate chemotaxis by differential activation of Rac1 and Rac2. *J Immunol* 2009;**183:**2718–2728.
41. Sorsa T, Tjaderhane L, Konttinen YT *et al*. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med* 2006;**38:**306–321.
42. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4- length fragments. *J Biol Chem* 1995;**270:**5872–5876.
43. Uitto VJ, Overall CM, McCulloch C. Proteolytic host cell enzymes in gingival crevice fluid. *Periodontol 2000* 2003;**31:**77–104.
44. Bildt MM, Bloemen M, Kuijpers-Jagtman AM, Von den Hoff JW. Collagenolytic fragments and active gelatinize complexes in periodontitis. *J Periodontol* 2008;**79:** 1704–1711.
45. Hatakeyama S, Yaegashi T, Oikawa Y *et al*. Expression patterns of adhesion molecules in junctional epithelium differs from that in order gingival epithelia. *J Periodont Res* 2006;**41:**322–328.
46. Ye P, Chapple CC, Kumar RK, Hunter N. Expression patterns of E-cadherin, involucrin, and connexin gap junction proteins in the lining epithelia of inflamed gingiva. *J Pathol* 2000;**192:**58–66.
47. Daniel C, Albrecht H, Lüdke A *et al*. Nestin expression in repopulating mesangial cells promotes their proliferation. *Lab Invest* 2008;**88:**387–397.