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ORIGINAL ARTICLE

# Development of a diagnostic algorithm in periodontal disease and identification of genetic expression patterns: A preliminary report

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periodontal disease

**Abstract** *Background/purpose:* To identify genetic expression patterns that can be used to define an appropriate diagnostic algorithm of clinical use in periodontal disease.
*Materials and methods:* Total RNA was extracted from 13 samples corresponding to normal human gingiva (NHG) and human gingiva affected by periodontal disease (PDHG). A comprehensive gene expression analysis was carried out by microarray analysis using Affymetrix Human Genome U133 plus 2.0 oligonucleotide arrays.
*Results:* Sixty-six probe sets (genes and expressed sequence tags — EST) overexpressed in all samples of one of the comparison groups, were used for the diagnostic algorithm. All samples, including an independent test sample, were correctly classified as normal or periodontally affected using the diagnostic algorithm. In addition, 2596 genes/EST were upregulated and 1542 genes/EST were downregulated in PDHG, with numerous gene functions impaired in PDHG, especially those related to the immune response, cell-cell junctions, and extracellular matrix remodeling.
*Conclusion:* Our study reveals differential gene expression profiles in NHG and PDHG. The proposed diagnostic algorithm could have clinical usefulness for differential diagnosis in periodontal disease.


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## Introduction

The periodontium consists of several tissues and structures that support and invest the tooth, including the gingiva, periodontal ligament, cementum, and alveolar bone.[1] The term, periodontal disease, commonly refers to an inflammatory disorder that affects the normal condition of the periodontium and might cause loss of supporting connective tissue and alveolar bone. In this context, this disease results in the formation of periodontal pockets, or deepened crevices, between the gingiva and tooth root, and severe periodontal disease can result in occasional pain and discomfort, impaired mastication, and irreversible tooth loss.[2] The main causes of periodontal disease are associated with the presence of dental plaque microorganisms, although several environmental insults, genetic disorders, and systemic diseases can play important roles.

The global prevalence of severe periodontal disease ranges from 10%~15%, although up to 90% may be affected by some forms of periodontal disease.[3] In addition to age and race, other known risk factors for periodontal disease include the body mass index,[4] stress, tobacco, diabetes, and nutrition.[5,6] Furthermore, limited research on the genetic component of adult periodontitis suggests that approximately one-half of cases may be heritable.[7]

Clinical manifestations of periodontal disease are multiple, ranging from mild bleeding of chronic gingivitis produced during simple tooth brushing, to aggressive periodontitis with tooth loss.[3] In this milieu, the existing clinical diagnosis of periodontal disease is based on visual, clinical, and radiographic assessments of periodontal tissues, rendering the diagnostic algorithm something difficult and imprecise to determine.

Numerous diagnostic algorithms were proposed, based on a clinical periodontal examination of the patient and complementary radiographic and medical laboratory tests. However, all these diagnostic approaches are subjective and highly dependent on the operator.

Several studies suggested an important genetic influence in periodontal disease. Although specific genetic markers were identified in uncommon juvenile forms of periodontitis, previous studies of specific genetic markers in adults with periodontitis were not encouraging, and knowledge of the pathogenesis and genes implicated in periodontal disease is still deficient.[8]

Within this framework, microarray techniques were proposed as a new diagnostic tool that could detect the presence of active disease, predict future disease progression, and evaluate the response to periodontal therapy, thereby improving the clinical management of periodontal patients. Using these methods, several authors previously determined the global gene expression profiles of several types of human cancer[9–11] and other multifactorial diseases[12,13] including periodontal disease.[14,15] Nonetheless, genes involved in different biologic processes form complex interaction networks,[16] and several studies are necessary to determine the exact roles of these genes in periodontal disease.

The present study focused on identifying genetic expression patterns that can be used to define an appropriate diagnostic algorithm of clinical use in aggressive periodontal disease.

## Materials and methods

### Tissue samples

To determine the global gene expression profile of normal human gingiva (NHG) and human gingiva affected by periodontal disease (PDHG), samples (six of NHG and seven of PDHG) were obtained. The average age of NHG donor patients was 44.6 years, and of patients with periodontal disease was 42.6 years. All samples corresponded to biopsies of $2 \times 2 \times 2$ mm from patients undergoing minor oral surgical procedures under local anesthesia. NHG samples were obtained from patients without inflammation or gingival bleeding and normal periodontal attachment, during teeth extraction for orthodontic reasons, or crown enlargement for prosthetic reasons. PDHG samples were extracted from patients with clinical gingival inflammation, bleeding on probing, and periodontal attachment loss of $>$ 4 mm clinically and radiologically diagnosed as aggressive periodontitis. In both cases, biopsy samples corresponded to gingival papillae that adhered to the root surface of the extracted tooth, or crown enlargement (NHG) or from the periodontally affected teeth (PDHG) during surgical periodontal therapy with a mucoperiosteal flap.

All patients provided informed consent for the use of donated tissues in this work. This study was approved by the local Research and Ethics Committees of the University of Granada. Experiments were undertaken with the understanding and written consent of each subject and according to ethical principles, including the World Medical Association, *Declaration of Helsinki*, and additional requirements of Spain.

### Comprehensive gene expression analysis by a microarray

Once a tissue biopsy was obtained, samples were immediately immersed in 5 volumes of RNAlater (Ambion Applied Biosystems, Austin, TX, USA) at $-20^{\circ}$C, to prevent RNA degradation. Subsequently, RNAlater was removed, and whole samples were sectioned into small pieces and disrupted using a tissue blender homogenizer (IKA T10 basic ULTRA-TURRAX, Staufen, Germany) in the presence of lysis buffer. Total RNA corresponding to both types of samples was extracted using the Qiagen RNeasy System (Qiagen, Mississauga, Ontario, Canada), following the manufacturer's recommendations. Total complementary (c)DNA was synthesized using a T7-polyT primer and reverse transcriptase (Superscript II, Life Technologies, Carlsbad, CA, USA), and labeled cRNA was synthesized by *in vitro* transcription with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY, USA). The labeled cRNA was hybridized using Human Genome U133 plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA), and absolute expression values were calculated and normalized from the scanned array using Affymetrix Microarray software.

### Analysis of the microarray expression data

To identify all probe sets (genes and expressed sequence tags - EST), the expressions of which were markedly higher in one of the two groups of samples (NHG or PDHG)

**Table 1** Genes/EST included in the diagnostic algorithm whose expression was higher (upregulated) or lower (downregulated) in all PDHG samples as compared to NHG. Uniqid: Affymetrix Human Genome U133 Plus 2.0 reference of this specific gene/EST. Mean expression data correspond to average expression for the group of NHG or PDHG expressed as Affymetrix fluorescent units (f.u.). FC = fold-change relative expression of the group of PDHG versus the group of NHG; NHG = normal human gingival; PDHG = human gingival affected by periodontal disease. P value corresponds to the significance level of the Student *t* test for the comparison of PDHG versus NHG.

| Status in PDHG | UNIQID | Gene symbol | Mean NHG | Mean PDHG | FC PDHG/ NHG | P | Gene title |
|---|---|---|---|---|---|---|---|
| Downregulated | 230443_at | NHP2L1 | 20.25 | 3.03 | 0.15 | 0.018 | NHP2 non-histone chromosome protein 2-like 1 (*S. cerevisiae*) |
| Downregulated | 1556147_at | — | 12.45 | 2.1 | 0.17 | 0.002 | CDNA clone IMAGE:5274427 |
| Downregulated | 219612_s_at | FGG | 8.42 | 1.62 | 0.19 | 0.011 | Fibrinogen gamma chain |
| Downregulated | 235426_at | GATM | 15.97 | 3.02 | 0.19 | 0.01 | Glycine amidinotransferase (L-arginine:glycine amidinotransferase) |
| Downregulated | 234448_at | — | 7.45 | 1.53 | 0.21 | 0.002 | MRNA; cDNA DKFZp434F1872 (from clone DKFZp434F1872) |
| Downregulated | 236903_at | — | 10.27 | 2.28 | 0.22 | 0.004 | Transcribed locus |
| Downregulated | 1560842_a_at | — | 8.68 | 2.97 | 0.34 | 0.003 | CDNA FLJ35846 fis, clone TESTI2006877 |
| Downregulated | 211196_at | DBT | 8.1 | 2.93 | 0.36 | 0.00042 | Dihydrolipoamide branched chain transacylase E2 |
| Downregulated | 230644_at | LRFN5 | 10.73 | 4.07 | 0.38 | 0.002 | Leucine rich repeat and fibronectin type III domain containing 5 |
| Downregulated | 232423_at | ARSD | 63.38 | 24.7 | 0.39 | 0.009 | Arylsulfatase D |
| Downregulated | 230948_at | — | 30.22 | 16 | 0.53 | 0.002 | CDNA FLJ42198 fis, clone THYMU2034338 |
| Downregulated | 219796_s_at | MUPCDH | 2.22 | 1.18 | 0.53 | 0.001 | Mucin-like protocadherin |
| Downregulated | 230585_at | — | 101.77 | 56.5 | 0.56 | 0.005 | Transcribed locus |
| Downregulated | 231725_at | PCDHB2 | 53.58 | 29.97 | 0.56 | 0.009 | Protocadherin beta 2 |
| Downregulated | 239863_at | — | 48.02 | 27.3 | 0.57 | 0.001 | CDNA FLJ43322 fis, clone NT2RI2027975 |
| Downregulated | 210202_s_at | BIN1 | 41.85 | 23.75 | 0.57 | 0.002 | Bridging integrator 1 |
| Downregulated | 1554068_s_at | FLJ32549 | 34.07 | 19.35 | 0.57 | 0.022 | Hypothetical protein FLJ32549 |
| Downregulated | 238518_x_at | GLYCTK | 33.12 | 18.98 | 0.57 | 0.008 | Glycerate kinase |
| Downregulated | 202789_at | PLCG1 | 116.63 | 67.9 | 0.58 | 0.004 | Phospholipase C, gamma 1 |
| Downregulated | 1556111_s_at | — | 26.82 | 15.95 | 0.59 | 0.051 | CDNA FLJ39819 fis, clone SPLEN2010534 |
| Downregulated | 244293_at | UQCRB | 26.73 | 15.78 | 0.59 | 0.001 | Ubiquinol-cytochrome c reductase binding protein |
| Downregulated | 232662_x_at | C10orf58 | 441.43 | 270.6 | 0.61 | 0.001 | Chromosome 10 open reading frame 58 |
| Downregulated | 226719_at | — | 87.03 | 57.6 | 0.66 | 0.002 | CDNA FLJ34899 fis, clone NT2NE2018594 |
| Downregulated | 220553_s_at | PRPF39 | 195.18 | 132.33 | 0.68 | 0.011 | PRP39 pre-mRNA processing factor 39 homolog (S. cerevisiae) |
| Downregulated | 239538_at | ZRANB3 | 40.37 | 27.52 | 0.68 | 0.005 | zinc finger, RAN-binding domain containing 3 |
| Downregulated | 224747_at | UBE2Q2 | 967.83 | 666.13 | 0.69 | 0.012 | Ubiquitin-conjugating enzyme E2Q (putative) 2 |
| Downregulated | 213573_at | — | 193.12 | 139.87 | 0.72 | 0.006 | Transcribed locus |
| Downregulated | 239376_at | — | 75.75 | 56.92 | 0.75 | 0.004 | CDNA clone IMAGE:4333081 |
| Downregulated | 207121_s_at | MAPK6 | 2951.82 | 2312.5 | 0.78 | 0.006 | Mitogen-activated protein kinase 6 |
| Downregulated | 214672_at | TTLL5 | 144.03 | 125.35 | 0.87 | 0.014 | Tubulin tyrosine ligase-like family, member 5 |
| Upregulated | 203380_x_at | SFRS5 | 949.47 | 1264.72 | 1.33 | 0.005 | Splicing factor, arginine/serine-rich 5 |
| Upregulated | 225715_at | KIAA1303 | 47.58 | 68.47 | 1.44 | 0.016 | Raptor |
| Upregulated | 223554_s_at | RANGRF | 17.1 | 30.67 | 1.79 | 0.006 | RAN guanine nucleotide release factor |
| Upregulated | 201340_s_at | ENC1 | 16.4 | 29.5 | 1.8 | 0.002 | Ectodermal-neural cortex (with BTB-like domain) |
| Upregulated | 243240_at | — | 13.88 | 25.72 | 1.85 | 0.00029 | Transcribed locus |
| Upregulated | 228804_at | DGCR5 | 12.17 | 24.27 | 1.99 | 0.00215 | DiGeorge syndrome critical region gene 5 (non-coding) |
| Upregulated | 232497_at | ZNF3 | 5.57 | 11.35 | 2.04 | 0.016 | Zinc finger protein 3 |
| Upregulated | 204535_s_at | REST | 6.27 | 14.42 | 2.3 | 0.017 | RE1-silencing transcription factor |
| Upregulated | 241054_at | — | 7.37 | 19.17 | 2.6 | 0.001 | Transcribed locus |

**Table 1** (*continued*)

| Status in PDHG | UNIQID | Gene symbol | Mean NHG | Mean PDHG | FC PDHG/ NHG | P | Gene title |
|---|---|---|---|---|---|---|---|
| Upregulated | 217143_s_at | TRA@ /// TRD@ | 20.08 | 53.68 | 2.67 | 0.012 | T cell receptor alpha locus ///T cell receptor delta locus |
| Upregulated | 1563341_at | — | 5.93 | 15.98 | 2.69 | 0.005 | CDNA clone IMAGE:4831161 |
| Upregulated | 1557816_a_at | — | 3.7 | 10.68 | 2.89 | 0.029 | Homo sapiens, clone IMAGE:3453782, mRNA |
| Upregulated | 244238_at | — | 5.97 | 18.57 | 3.11 | 0.017 | Transcribed locus, moderately similar to XP_001137143.1 |
| Upregulated | 1560550_at | — | 13.18 | 41.75 | 3.17 | 0.00022 | Homo sapiens, clone IMAGE:5180210, mRNA |
| Upregulated | 243864_at | CCDC80 | 4.03 | 13.6 | 3.37 | 0.001 | Coiled-coil domain containing 80 |
| Upregulated | 233971_at | LOC401565 | 4.45 | 15.03 | 3.38 | 0.001 | Similar to 4931415M17 protein |
| Upregulated | 241853_at | — | 2.3 | 8.05 | 3.5 | 0.038 | Transcribed locus |
| Upregulated | 244036_at | — | 3.22 | 11.47 | 3.56 | 0.037 | Transcribed locus |
| Upregulated | 213955_at | MYOZ3 | 2.98 | 10.65 | 3.57 | 0.018 | Myozenin 3 |
| Upregulated | 238429_at | TMEM71 | 6.73 | 25.02 | 3.72 | 0.00023 | Transmembrane protein 71 |
| Upregulated | 229179_at | RUFY1 | 1.68 | 6.82 | 4.05 | 0.015 | RUN and FYVE domain containing 1 |
| Upregulated | 237220_at | — | 1.52 | 6.23 | 4.11 | 0.069 | Transcribed locus |
| Upregulated | 240065_at | FAM81B | 2.22 | 9.18 | 4.14 | 0.002 | Family with sequence similarity 81, member B |
| Upregulated | 1554886_a_at | MLXIP | 2.53 | 10.75 | 4.24 | 0.032 | MLX interacting protein |
| Upregulated | 1560943_s_at | — | 4.1 | 18.67 | 4.55 | 0.007 | — |
| Upregulated | 220565_at | CCR10 | 2.87 | 13.38 | 4.67 | 0.011 | Chemokine (C-C motif) receptor 10 |
| Upregulated | 213965_s_at | CHD5 | 2.45 | 13.03 | 5.32 | 0.047 | Chromodomain helicase DNA binding protein 5 |
| Upregulated | 216096_s_at | NRXN1 | 2.07 | 11.12 | 5.38 | 0.036 | Neurexin 1 |
| Upregulated | 231287_s_at | Klkbl4 | 2.22 | 11.98 | 5.41 | 0.064 | Plasma kallikrein-like protein 4 |
| Upregulated | 210013_at | HPX | 3.87 | 21.98 | 5.69 | 0.048 | Hemopexin |
| Upregulated | 243836_at | KIAA1843 | 0.75 | 4.3 | 5.73 | 0.075 | KIAA1843 protein |
| Upregulated | 1564134_at | — | 1.77 | 11.15 | 6.31 | 0.017 | Transcribed locus |
| Upregulated | 1556564_at | HHIPL1 | 2.28 | 15.78 | 6.91 | 0.006 | HHIP-like 1 |
| Upregulated | 232629_at | PROK2 | 4.42 | 35.08 | 7.94 | 0.055 | Prokineticin 2 |
| Upregulated | 207327_at | EYA4 | 1.53 | 12.33 | 8.04 | 0.001 | Eyes absent homolog 4 (Drosophila) |
| Upregulated | 222202_at | LOC644213 | 0.78 | 9.08 | 11.6 | 0.020 | Similar to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) |

compared to the other group, we first calculated the average expression of each gene/EST in each sample group (NHG and PDHG) using six samples of each type. Then, the relative multiple of change in the expression of PDHG versus NHG samples was calculated, and all genes/EST with a relative multiple change in expression of $> 2$ were selected as being overexpressed in PDHG. Genes with a change in the relative multiple of expression of $< 0.5$ were selected as being downregulated in PDHG. A Gene Ontology (GO) analysis of the selected genes/EST was performed using BiNGO (http://www.psb.ugent.be/cbd/papers/BiNGO/), a plug-in for the program Cytoscape.[17] The set of selected genes was tested for enrichment of any GO category relating to "biological processes" compared to all annotated genes represented on the array. Scores were evaluated based on the hypergeometric distribution and Bonferroni correction for multiple testing. Thus, the P value reflects the likelihood that one observed such enrichment by chance alone.[18]

To develop a periodontal disease diagnostic algorithm, we identified and selected all genes/EST with absolute expression levels higher for each of the six samples of one group (NHG or PDHG) than for each of the six samples of the other group. No exceptions were allowed. Average values of each of these selected genes were statistically compared using Student *t* test. Values of $P < 0.05$ were considered statistically significant for two-tailed comparisons.

Hierarchical clustering was performed on the 12 samples used to develop the classification algorithm using Cluster and Tree-View software (Stanford University, Palo Alto, CA, USA). Datasets used for hierarchical clustering were normalized by standardizing each expression level of each gene/EST and each sample to a mean of 0 and variance of 1. To validate the algorithm, the 12 samples were clustered along with an independent sample corresponding to a PDHG biopsy. All cluster analyses were performed in a blinded manner; that is, the sample type corresponding to each sample was decoded only at the end of the process.

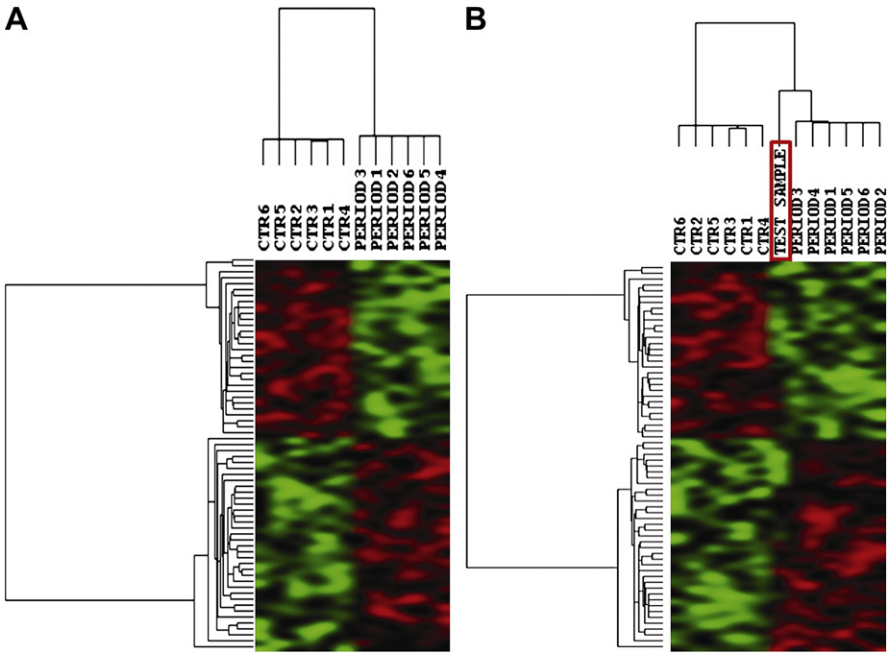


**Figure 1** Proposed diagnostic algorithm for periodontal research. (A) Dendrogram representing cluster analysis of the 12 samples used for developing the diagnostic algorithm; (B) cluster analysis of the 12 samples and 1 additional test PDHG sample used to validate the algorithm. Genes with high relative expression are shown in red and genes with expression below the average are shown in green. CTR = control NHG samples; PERIOD = PDHG samples; Test sample = periodontally affected sample used to validate the algorithm.

### Protein expression by Western blotting

Whole-protein extracts of samples corresponding to NHG and PDHG were obtained at the same experimental time of messenger (m)RNA isolation using the Qiagen RNeasy System (Qiagen). To perform this, 70% ethanol was added to cell lysates and placed on an RNeasy mini column. All of the flow-through was collected to obtain whole proteins from the samples following the manufacturer's instructions. Therefore, 20 μL of each protein extract was loaded on NuPAGE Novex 10% Bis-Tris gels (ref. NP0301, Invitrogen, Carlsbad, CA, USA), separated by electrophoresis, and transferred to nitrocellulose membranes following standard procedures. Membranes were then blocked for 1 hour with 5% skimmed milk, before overnight incubation with the following primary antibodies: rabbit anti-arylsulfatase D (ARSD) (1:250, Sigma-Aldrich ref. HPA004694, St. Louis, MO, USA), mouse anti-MAPK6 (1:200, Sigma-Aldrich ref. WH0005597M1), and mouse anti-GAPDH (1:300, Calbiochem-Merck ref. CB1001, KGaA, Darmstadt, Germany). Anti-ARSD and anti-MAPK6 antibodies were selected from the list of significant genes found by the microarray analysis, among all genes with significant differences between NHG and PDHG for which an antibody was commercially available. Primary antibodies were detected using a horseradish peroxidase-linked anti-mouse immunoglobulin G (IgG) (Sigma-Aldrich ref. A9044) or anti-rabbit IgG (Sigma-Aldrich ref. A0545). Visualization was performed using the ECL Western Blotting Detection Reagent (GE Healthcare, Waukesha, WI, USA). Blots were exposed to autoradiography Kodak Biomax XAR films (Rochester, NY, USA).

## Results

### Development of a diagnostic algorithm for periodontal disease

The statistical analysis of all genes analyzed by the microarray analysis showed that expressions of 66 genes/EST were significantly higher in all samples of a specific group (NHG or PDHG). Indeed, 30 genes/EST were more highly expressed in all NHG samples, whereas 36 genes/EST were more highly expressed in all PDHG samples [$P < 0.001$ (Table 1)]. Of the 30 genes/EST downregulated in PDHG, one-third of the genes/EST were expressed by at least a 2-fold greater extent in control NHG than in diseased samples. By contrast, 83.3% of the selected genes were upregulated by at least 2-fold in PDHG compared to NHG.

The analysis of those 66 genes/EST revealed that the functions of the genes/EST upregulated in the two groups were partially similar. However, two genes with an important role in the activation and regulation of the immune response [the T cell receptor alpha and delta locus (TRA$\alpha$ and TRD$\alpha$), and the chemokine C-C motif receptor 10; CCR10] were only upregulated in PDHG samples, whereas a gene related to physiological extracellular matrix (ECM) remodeling (ARSD) and a gene with a function in tissue regeneration (L-arginine-glycine amidinotransferase; GATM) were overexpressed only in control NHG.

When all 66 genes/EST selected were used to classify samples analyzed in this work, we found that all 12 samples were correctly clustered, with the six normal samples clustered in one of the main branches of the tree and the

remaining samples in the other branch [Fig. 1(A)]. Strikingly, validation of the classification algorithm using an independent validation sample corresponding to a PDHG sample showed that this test sample was properly clustered in the group of periodontally affected tissues [Fig. 1(B)].

### Identification of genes significantly overexpressed by NHG and PDHG

The gene expression analysis of NHG and PDHG revealed that 1542 genes/EST were downregulated in PDHG by at least 2-fold, whereas 2596 genes/EST were upregulated in PDHG compared to control NHG samples (Supplementary Table S1). An analysis of gene functions of these selected genes using Cytoscape-BiNGO showed a statistically significant enrichment of four gene functions in the NHG group, with 32 gene functions significantly represented by PDHG (Table 2). Among the four gene functions that were more represented by NHG, three functions corresponded to the synthesis of several structural cell constituents, and one was related to cell and tissue development. By contrast, functions overrepresented by PDHG samples included 10 functions related to the immune response or responses to different stimuli, nine functions to structural cell components, three functions to the ECM, seven functions to signal transduction and cell physiology, one function to development, and one function to cell-cell adhesion. Illustrative gene function diagrams corresponding to NHG and PDHG are given in Figs. 3 and 4, respectively.

### Western blot analysis of relevant genes in NHG and PDHG

As shown in Fig. 2, the Western blot analysis of NHG and PDHG cell lysates demonstrated that MAPK6 protein expression was higher in NHG than in PDHG, as was the case for the microarray analysis. Similarly, the ARSD protein was significantly overexpressed in NHG samples compared to PDHG samples.

**Table 2** Analysis of Gene-Ontology gene functions of genes significantly upregulated in NHG and in PDHG using Cytoscape-BiNGO. GO-ID = Gene-Ontology reference for the specific function. P-value = statistical significance value for the hypergeometric distribution. The group of samples (NHG or PDHG) in which each significant function was overrepresented is shown in the last column of the table.

| GO-ID | P | Gene functions | Upregulated in |
|---|---|---|---|
| 7275 | 0.004 | Development | NHG |
| 16020 | 0.039 | Membrane | NHG |
| 5886 | 0.00010 | Plasma membrane | NHG |
| 44459 | 0.018 | Plasma membrane part | NHG |
| 3823 | 0.005 | Antigen binding | PDHG |
| 7155 | 0.00001 | Cell adhesion | PDHG |
| 7154 | 0.00000 | Cell communication | PDHG |
| 7267 | 0.022 | Cell-cell signaling | PDHG |
| 5575 | 0.00002 | Cellular component | PDHG |
| 6952 | 0.00000 | Defense response | PDHG |
| 7275 | 0.00005 | Development | PDHG |
| 31012 | 0.006 | Extracellular matrix | PDHG |
| 5201 | 0.008 | Extracellular matrix structural constituent | PDHG |
| 5576 | 0.00000 | Extracellular region | PDHG |
| 6959 | 0.017 | Humoral immune response | PDHG |
| 6955 | 0.00000 | Immune response | PDHG |
| 16021 | 0.00000 | Integral to membrane | PDHG |
| 5887 | 0.00012 | Integral to plasma membrane | PDHG |
| 31224 | 0.00000 | Intrinsic to membrane | PDHG |
| 31226 | 0.00010 | Intrinsic to plasma membrane | PDHG |
| 16020 | 0.00000 | Membrane | PDHG |
| 44425 | 0.00000 | Membrane part | PDHG |
| 50874 | 0.00000 | Organismal physiological process | PDHG |
| 5886 | 0.00000 | Plasma membrane | PDHG |
| 44459 | 0.00005 | Plasma membrane part | PDHG |
| 4872 | 0.00000 | Receptor activity | PDHG |
| 5102 | 0.006 | Receptor binding | PDHG |
| 9607 | 0.00000 | Response to biotic stimulus | PDHG |
| 9605 | 0.00002 | Response to external stimulus | PDHG |
| 51707 | 0.00023 | Response to other organism | PDHG |
| 9613 | 0.00013 | Response to pest, pathogen or parasite | PDHG |
| 50896 | 0.00000 | Response to stimulus | PDHG |
| 9611 | 0.006 | Response to wounding | PDHG |
| 4871 | 0.00000 | Signal transducer activity | PDHG |
| 7165 | 0.00003 | Signal transduction | PDHG |
| 4888 | 0.00019 | Transmembrane receptor activity | PDHG |

## Discussion

Periodontal disease is a chronic, destructive condition affecting a high percentage of the adult population, characterized by a persistent oral bacterial infection which replaces the normal microbiota.[19] This may be due to disequilibrium in the host, which can be caused by several factors, such as genetic factors, modification of the environmental conditions of the infected site, a significant decrease in the proportion of beneficial bacteria producing inhibitory substances, and/or decreased efficacy of the host immune system.[20,21] There is no doubt that genes play an important role in the predisposition to and progression of periodontal diseases.[22]

Although the disease is very prevalent and often aggressive, diagnostic methods currently available for this pathology are mainly subjective, and a firm, objective, and observer-independent diagnostic algorithm has not been established to date.[23,24] Moreover, elucidating the manner and extent to which genetic factors contribute to the origin and progression of this disease, may have important therapeutic implications. In this context, high-throughput, genome-wide analytical technologies, especially expression microarrays, were shown to be both accurate and precise when properly implemented.[25] Expression microarrays can be used for disease classification and identification of causal mechanisms in different human diseases,[25] including multiple sclerosis,[26] cancer,[27] and

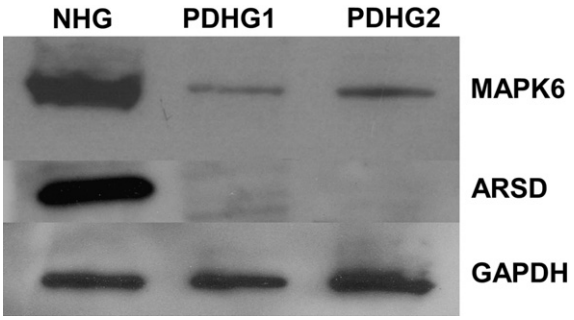


**Figure 2** Western blot analysis of ARSD (arylsulfatase D) and MAPK6 (mitogen-activated protein kinase 6) protein expression in control NHG and two illustrative PDHG samples. Expression of the housekeeping protein GAPDH was used as a control of initial protein loading.

even periodontal disease.[14,28] However, a microarray-based diagnostic algorithm had not been proposed to date.

In this work, we were able to identify a number of genes and gene functions, the expressions of which were altered in PDHG compared to NHG. In the first place, our gene-function analysis demonstrated that numerous gene functions related to the synthesis of some structural components and cell physiological processes were significantly altered in PDHG. These results suggest that extensive alteration of cell functions takes place in tissues affected by aggressive periodontal disease. Furthermore, our analysis revealed downregulation of genes with a role in maintaining a structural cell membrane barrier in PDHG compared to control NHG.

In the second place, our gene-expression analysis revealed that a high number of genes associated with the immune response were impaired in PDHG samples, including the TRA$\alpha$ and CCR10 genes. The TRA$\alpha$/TRD$\alpha$ is expressed on the surface of T lymphocytes and is responsible for recognizing antigens bound to the major histocompatibility complex (MHC). This gene was also demonstrated to be an essential mediator of alveolar bone destruction in rapidly progressing cases.[29] This could explain why periodontal disease is characterized by alveolar bone loss and immune-system activation. On the other hand, chemokine receptors such as CCR10 may play a fundamental role in activating the immune system, cell trafficking of various types of leukocytes, homeostasis, and angiogenesis. In this milieu, gums and pocket bleeding, early signs of the periodontal disease, could be predicted early by chemokine upregulation tests designed as a microarray. In addition, much research suggested that bacterial colonization may lead to an altered environment, where overexpression of TRA$\alpha$ is stimulated by inflammatory cytokines such as CCR10 in response to infection.[30]

Additionally, it is well known that cell-cell junctions can be altered in periodontal disease by bacterial colonization and protease activity mainly caused by *Porphyromonas gingivalis*, which is able to degrade several kinds of epithelial junctional proteins.[31,32] Our microarray analysis confirmed this cell-cell junction alteration in PDHG, with

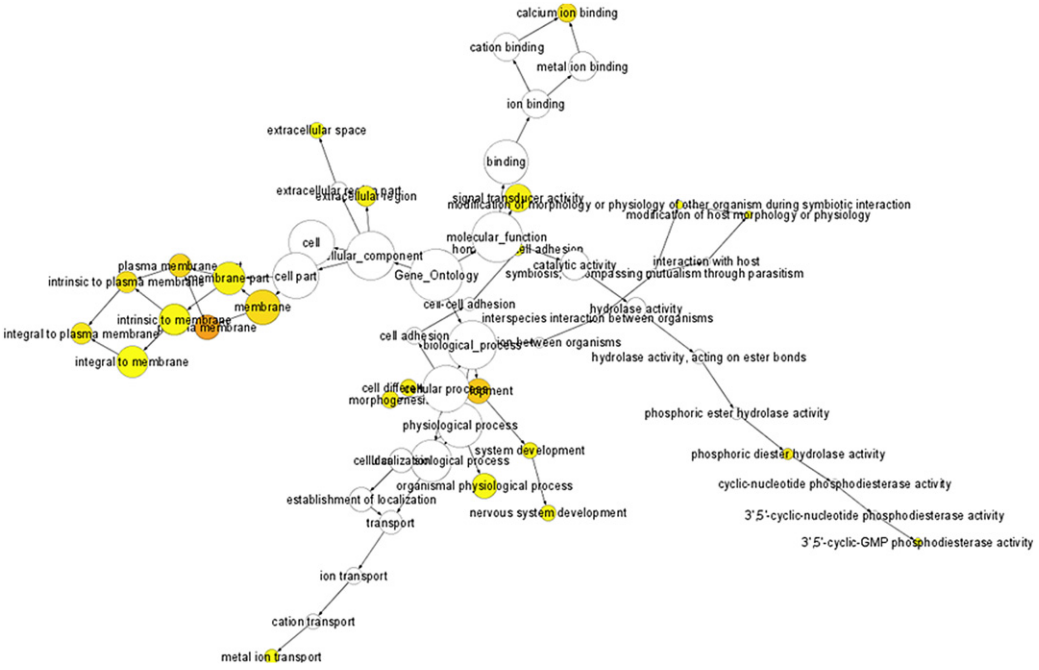


**Figure 3** Significant results from the Gene-Ontology analysis of genes upregulated in NHG using Cytoscape-BiNGO are shown within their hierarchical tree of the Gene-Ontology classification system. Each node represents a single Gene-Ontology category. Starting from the most general category, genes are classified stepwise into smaller and more specific categories, which are shown as daughter branches. Branches that were not enriched in our analysis are not shown. The number of genes in each Gene-Ontology category is represented by the node size and the statistical enrichment (P value from a hypergeometric distribution test) of these genes is represented as pseudocolor (red for highly enriched, orange for significantly enriched, yellow for potentially enriched and white for non-enriched).

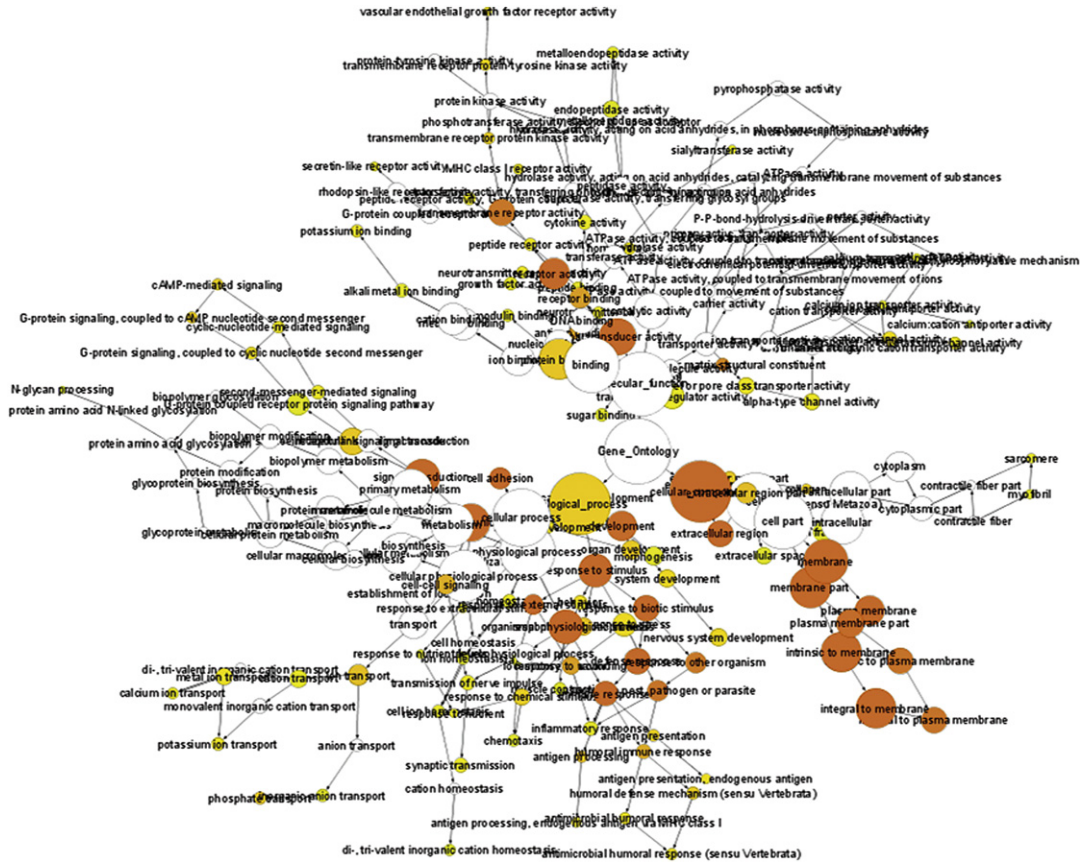


**Figure 4** Significant results from the Gene-Ontology analysis of genes upregulated in PDHG using Cytoscape-BiNGO are shown within their hierarchical tree of the Gene-Ontology classification system. Each node represents a single Gene-Ontology category. Starting from the most general category, genes are classified stepwise into smaller and more specific categories, which are shown as daughter branches. Branches that were not enriched in our analysis are not shown. The number of genes in each Gene-Ontology category is represented by the node size and the statistical enrichment (P value from a hypergeometric distribution test) of these genes is represented as pseudocolor (red for highly enriched, orange for significantly enriched, yellow for potentially enriched and white for non-enriched).

significant changes in the expressions of cadherins (CDH2, CDH7, and CDH16), junctophilins (JPH2 and JPH3), and a tight junction protein (TJP3). All of these junction proteins are characterized by their relevant contributions to stabilization of intercellular junctions, and any modification of their functions may alter the crucial barrier function of the oral-mucosa epithelium.[33]

Finally, altered expressions of genes related to the synthesis and remodeling of the ECM such as ARSD, several types of collagens (COL4A6, COL11A1, COL14A1, COL24A1, COL25A1, and COL9A1), and type III fibronectin (FSD1L) in PDHG suggest that the capacity of PDHG tissues to remodel and repair components of the ECM is impaired in this disease.

Although this study has several limitations, including the low number of samples analyzed, the possibility of false negatives or false positives, and the limited value of genetic patterns as predictors of periodontal disease per se, from the results of this study, we proposed a preliminary diagnostic algorithm based on the microarray-determined gene expressions of a reduced number of relevant genes/EST. Using this algorithm, it was possible to properly classify the 12 samples used to develop the algorithm, and also to correctly diagnose an independent sample corresponding to diseased PDHG. Although some previous studies significantly contributed to a better understanding of the pathobiology of this disease,[14,28] this is the first time that an algorithm based on a microarray gene-expression analysis allows for an observer-independent diagnosis of human periodontal disease. Using this novel technique will contribute to a better understanding of the genetic mechanisms that are involved in the genesis and progression of human aggressive periodontal disease, and also will allow the accurate classification of a patient as normal or periodontally affected in a completely objective way. This will be especially useful in cases in which clinical signs and symptoms are not clear, in order to establish appropriate treatment.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jds.2012.01.007.

## References


1. Lin NH, Gronthos S, Bartold PM. Stem cells and periodontal regeneration. *Aust Dent J* 2008;53:108–21.
2. Greenwell H. Position paper: guidelines for periodontal therapy. *J Periodontol* 2001;72:1624–8.
3. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005;366:1809–20.
4. Genco RJ, Grossi SG, Ho A, Nishimura F, Murayama Y. A proposed model linking inflammation to obesity, diabetes, and periodontal infections. *J Periodontol* 2005;76:2075–84.
5. Pinson M, Hoffman WH, Garnick JJ, Litaker MS. Periodontal disease and type I diabetes mellitus in children and adolescents. *J Clin Periodontol* 1995;22:118–23.
6. Bagaitkar J, Demuth DR, Scott DA. Tobacco use increases susceptibility to bacterial infection. *Tob Induc Dis* 2008;4:12.
7. Michalowicz BS, Diehl SR, Gunsolley JC, et al. Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol* 2000;71:1699–707.
8. Kornman KS, di Giovine FS. Genetic variations in cytokine expression:a risk factor for severity of adult periodontitis. *Ann Periodontol* 1998;3:327–38.
9. Mora J, Alaminos M, de Torres C, et al. Comprehensive analysis of the 9p21 region in neuroblastoma suggests a role for genes mapping to 9p21-23 in the biology of favourable stage 4 tumours. *Br J Cancer* 2004;91:1112–8.
10. Martinez R, Martin-Subero JI, Rohde V, et al. A microarray-based DNA methylation study of glioblastoma multiforme. *Epigenetics* 2009;4:255–64.
11. Santos ES, Blaya M, Raez LE. Gene expression profiling and non-small-cell lung cancer:where are we now? *Clin Lung Cancer* 2009;10:168–73.
12. Borsy A, Podani J, Steger V, et al. Identifying novel genes involved in both deer physiological and human pathological osteoporosis. *Mol Genet Genomics* 2009;281:301–13.
13. Bauer JW, Bilgic H, Baechler EC. Gene-expression profiling in rheumatic disease:tools and therapeutic potential. *Nat Rev Rheumatol* 2009;5:257–65.
14. Beikler T, Peters U, Prior K, Eisenacher M, Flemmig TF. Gene expression in periodontal tissues following treatment. *BMC Med Genomics* 2008;1:30.
15. Tanke HJ. Genomics and proteomics:the potential role of oral diagnostics. *Ann N Y Acad Sci* 2007;1098:330–4.
16. Covani U, Marconcini S, Giacomelli L, Sivozhelevov V, Barone A, Nicolini C. Bioinformatic prediction of leader genes in human periodontitis. *J Periodontol* 2008;79:1974–83.
17. Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* 2005;21:3448–9.
18. Boyer LA, Plath K, Zeitlinger J, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 2006;441:349–53.
19. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. *Periodontol 2000* 1997;14:12–32.
20. Gendron R, Grenier D, Maheu-Robert L. The oral cavity as a reservoir of bacterial pathogens for focal infections. *Microbes Infect* 2000;2:897–906.
21. Gorska R, Gregorek H, Kowalski J, Laskus-Perendyk A, Syczewska M, Madalinski K. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. *J Clin Periodontol* 2003;30:1046–52.
22. Gera I, Vari M. Genetic background of periodontitis. Part I. Basic principles and inherited syndromes. Literature review. *Fogorv Sz* 2009;102:87–95 [in Hungarian, English abstract].
23. Kinane DF, Hart TC. Genes and gene polymorphisms associated with periodontal disease. *Crit Rev Oral Biol Med* 2003;14:430–49.
24. Savage A, Eaton KA, Moles DR, Needleman I. A systematic review of definitions of periodontitis and methods that have been used to identify this disease. *J Clin Periodontol* 2009;36:458–67.
25. Bhattacharya S, Mariani TJ. Array of hope:expression profiling identifies disease biomarkers and mechanism. *Biochem Soc Trans* 2009;37:855–62.
26. Steinman L, Zamvil S. Transcriptional analysis of targets in multiple sclerosis. *Nat Rev Immunol* 2003;3:483–92.
27. Van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347:1999–2009.
28. Demmer RT, Behle JH, Wolf DL, et al. Transcriptomes in healthy and diseased gingival tissues. *J Periodontol* 2008;79:2112–24.
29. Gao X, Teng YT. T-cell-receptor gene usage of Actinobacillus actinomycetemcomitans-reactive periodontal CD4+ T cells from localized juvenile periodontitis patients and human peripheral blood leukocyte-reconstituted NOD/SCID mice. *J Periodontal Res* 2002;37:399–404.
30. Amanuma R, Nakajima T, Yoshie H, Yamazaki K. Increased infiltration of CD1d and natural killer T cells in periodontal disease tissues. *J Periodontal Res* 2006;41:73–9.
31. Kuramitsu HK. Proteases of Porphyromonas gingivalis:what don't they do? *Oral Microbiol Immunol* 1998;13:263–70.
32. Katz J, Yang QB, Zhang P, et al. Hydrolysis of epithelial junctional proteins by Porphyromonas gingivalis gingipains. *Infect Immun* 2002;70:2512–8.
33. Amano A. Disruption of epithelial barrier and impairment of cellular function by Porphyromonas gingivalis. *Front Biosci* 2007;12:3965–74.