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ORIGINAL ARTICLE

Virulence genes of *Porphyromonas gingivalis* W83 in chronic periodontitis

LI LIN, CHEN LI, JINGBO LIU, DONGMEI ZHANG, JIAN ZHAO, YURONG KOU, NING YU & YAPING PAN

Department of Periodontics, School of Stomatology, China Medical University, Shenyang, China

Abstract

Objective. To identify virulence genes found in highly virulent strains of *Porphyromonas gingivalis* (*P. gingivalis*) among Chinese patients with chronic periodontitis and to evaluate the association of these virulence genes with clinical parameters and with periodontal tissue destruction. ***Material and methods.*** Suppression subtractive hybridization was applied to acquire short gene fragments harbored only in virulent strains of *P. gingivalis* W83. Eighteen genes, which were present in *P. gingivalis* W83 but absent from *P. gingivalis* ATCC 33277, were labeled with Cy5 and used as probes in DNA microarray hybridization to analyze DNA of *P. gingivalis* isolated from chronic periodontitis patients. ***Results.*** Spearman correlation analysis revealed 10 genes correlated with probing depth, clinical attachment loss, and tooth mobility ($p < 0.05$). ***Conclusion.*** These genes may provide an important clue towards our understanding the mechanism of occurrence and the development of periodontal disease.

Key Words: *DNA, microarray, suppression subtractive hybridization*

Introduction

Chronic periodontitis is an inflammatory disease of the supporting tissues of teeth involving alveolar bone resorption, which can lead to eventual tooth loss [1]. The presence of a consortium of Gram-negative bacteria in subgingival plaque has been associated with the development of chronic periodontitis. In this consortium, *Porphyromonas gingivalis* has been identified as a major pathogen [2].

P. gingivalis, a Gram-negative, anerobic, black-pigmented bacterium, is considered to be a primary etiologic agent of certain periodontal diseases [3]. *P. gingivalis* was etiologically associated with various types of periodontal disease, including chronic periodontitis (CP). This bacterium is frequently detected in deep periodontal pockets of CP patients and is occasionally found in healthy periodontal tissues without inflammation [4]. It has been suggested that diversity in virulence exists among the organisms harbored by individuals with healthy periodontal tissues and those with periodontitis [5].

P. gingivalis has been classified into virulent and avirulent strains based on its ability to form necrotic abscesses in animal models. It has been suggested that the ability to form necrotic abscesses of virulent *P. gingivalis* is important in the initiation and progression of periodontal disease [3]. Strains of *P. gingivalis* have been differentiated by restriction fragment length polymorphism analysis of insertion sequences [6], and by heteroduplex and PCR analysis of the ribosomal intergenic spacer region [7]. The genome sequence of a virulence *P. gingivalis* strain W83 has recently been completed [8]. Apparently, there are virulence differences between *P. gingivalis* strains, but the mechanisms underlying these differences are not fully understood. To obtain more information about pathogenicity and virulence of *P. gingivalis*, it is necessary to assess the genetic population structure of the species and to examine the occurrence of putative virulence genes among clinical isolates. A number of virulence factors are known for *P. gingivalis*, including fimbriae, lipopolysaccharide, collagenase, and cysteine proteinases with trypsin-like activity [9,10]. These factors may contribute to some of the important clinical signs of periodontitis, including neutrophil accumulation at

Correspondence: Yaping Pan, Department of Periodontics, School of Stomatology, China Medical University, Nanjing North St. No.117, Shenyang, Liaoning, P.R. China 110002. Tel: +86 24 22891701. Fax: +86 24 22892645. E-mail: yppan@mail.cmu.edu.cn.

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infected sites and pocket deepening and gingival bleeding on probing [11].

As many as 100 different clonal types of *P. gingivalis* have been found in periodontitis patients [12,13]. Pathologic changes associated with chronic infection in progressing periodontitis lesions may accentuate differences between clonal types. Such differences could be caused by a variety of genetic mechanisms, including genetic exchange and recombination, insertion sequence (IS) movement and related activities, and recombination events involving non-mobile repeated DNA sequences.

In this study, we sought to identify virulence genes found in highly virulent strains of *P. gingivalis* among Chinese patients with chronic periodontitis and to evaluate the correlation of these virulence genes with clinical parameters and with periodontal tissue destruction.

Material and methods

Bacterial strains and culture conditions

The *P. gingivalis* virulent strain W83 and the avirulent strain ATCC 33277 were used in this study. *P. gingivalis* strains were cultured in GAM broth (Nissui Seiyaku, Tokyo, Japan) supplemented with hemin (5 μg/ml) and vitamin K (1 μg/ml) at 37°C in a glove box (Model ANX-1; Hirasawa Works, Tokyo, Japan) containing 80% N_2, 10% H_2, and 10% CO_2 for 24 to 48 h. Subgingival plaque samples were inoculated into brain heart infusion blood agar plates (BHI; Difco Laboratories, Detroit, Mich., USA) supplemented with hemin (5 μg/ml), vitamin K (0.5 μg/ml), cysteine (0.1%), and 5% (v/v) sheep blood. The bacteria from the subgingival plaque samples were grown in the glove box at 37°C for 5 days. *P. gingivalis* colonies were identified by Gram-staining and morphological examination, biochemical reaction and *P. gingivalis* specific 16S rDNA gene detection by PCR. *Escherichia coli* JM109 (Takara, Dalian, China) was used for cloning. JM109 was cultured aerobically in Luria-Bertani (LB) medium (Takara), or in LB supplemented with ampicillin at 50 μg/ml after transformation with plasmid pMD-18T (Takara) and derivatives for cloning and sequencing.

Suppression subtractive hybridization (SSH)

Chromosomal DNA was purified (DNeasy Plant Mini Kit; Qiagen, Hilden, Germany) from *P. gingivalis* W83 (tester) and *P. gingivalis* ATCC 33277 (driver). The SSH protocol was based on instructions of the PCR-select bacterial genome subtraction kit (BD Bioscience Clontech, Palo Alto, Calif., USA). Briefly, total DNA from *P. gingivalis* ATCC 33277 (2 μg) and *P. gingivalis* W83 (2 μg) was digested overnight at 37°C with Rsa I restriction endonuclease (Promega Co., Madison, Wisc., USA). Tester DNA (100 ng) from *P. gingivalis* W83 was ligated in two separate reactions of 10 μl each with either adaptor 1 or 2R. Then 1 μl of each ligation product was heat denatured and separately hybridized to an excess of driver (600 ng of Rsa I-digested DNA from *P. gingivalis* ATCC 33277) for 1.5 h at 63°C. The two hybridization mixtures were then combined in the presence of 300 ng of driver DNA and incubated overnight at 63°C. Hybrids carrying both adaptors 1 and 2R were amplified by nested PCR in accordance with the manufacturer's instructions using *Taq* Advantage cDNA Polymerase Mix (BD Bioscience Clontech, Palo Alto, Calif., USA). The SSH PCR products (4 μl) were ligated overnight at 16°C with 3 units of T4 DNA ligase (Takara) in ligation buffer containing 50 ng of pMD18-T plasmid vector (Takara). Ligation products were used to transform *E. coli* strain JM109. Ampicillin-resistant white colonies were randomly picked and grown in LB broth containing ampicillin (50 μg/ml). Positive clones were identified by PCR and then sequenced. DNA sequencing was done by Invitrogen Biotechnology Co. Ltd. (Shanghai, China). Sequence data were analyzed using the BLAST database from the National Center for Biotechnology Information (NCBI). *P. gingivalis* ATCC 33277 or *P. gingivalis* W83 DNA (1 μg) was digested with Rsa I restriction endonuclease and 100 ng of the digested DNA was spotted on the Nytran Super Charge nylon N+transfer membrane (0.45 μm pore size; Schleicher & Schuell, Keene, N.H., USA). The unique DNA fragments isolated from SSH were labeled with 1 nmol of digoxigenin-11-dUTP by random priming (DIG High Prime kit; Roche, Mannheim, Germany), and used to hybridize with the chromosomal DNA of strain W83 and ATCC 33277. After washing, the probe was detected using anti-digoxigenin Fab fragment and CDP-Star as a substrate according to the DIG DNA labeling and detection Kit (Roche, Mannheim, Germany).

Clinical sample preparation

The subjects were 41 Chinese CP patients [23 M aged 35 to 66 years, mean (SD) age 45.7 (8.1) years; 18 F aged 37 to 65 years, mean (SD) age 44.3 (7.9) years] and 76 periodontal healthy individuals [39 M aged 28 to 48 years, mean (SD) age 35.7 (6.3) years; 37 F aged 31 to 52 years, mean (SD) age 37.2 (5.8) years] who were referred to the dental clinic in the Hospital School of Stomatology, China Medical University for dental or periodontal treatment or health monitoring. All the subjects were non-smokers with no systemic disease and with at least 14 teeth remaining. Those who had received professional cleaning or had a history of antibiotic therapy during the preceding 3 months were excluded. All of the

patients and the healthy individuals underwent full-mouth examination. The criteria of diagnosis for chronic periodontitis were based on the Classification of Periodontal Diseases issued by the American Academy of Periodontology in 1999 [14]. Briefly, the patients had >30% sites showing periodontal probing depth ≥3 mm, clinical attachment loss >1 mm, and radiographic evidence of alveolar bone loss. These individuals were considered periodontal healthy with periodontal probing depth <3 mm, without clinical attachment loss, with no inflammation of gingiva and no alveolar bone absorption on X-ray examination. All the subjects received detailed information concerning the nature of the study and the procedures involved, and their informed consent was obtained. The study was approved by the local ethics committee of the School of Stomatology of the China Medical University.

For each patient, each subgingival plaque sample was taken from the bottom of the periodontal pocket of the deepest sites of the 1st molar with separate sterile Gracy curettes after supragingival plaque was gently removed. For each periodontal healthy individual, each sample from the bottom of gingival sulcular of the 1st molar was collected with the same method and than transferred to a microcentrifuge tube containing 200 μl TE buffer (10 mmol/l Tris-HCl, 1.0 mmol/l EDTA, pH 8.0) and stored at −70°C.

A single examiner measured and recorded clinical parameters, which included sulcus bleeding index (SBI, assigning a score of 0–5, with 0 indicating a healthy appearance and no bleeding) [15], clinical attachment loss (CAL, measured in mm from the cemento-enamel junction to the base of the periodontal pocket, the CP sites showing CAL >1 mm), probing depth (PD, the distance from the free gingival margin to the base of the sulcus/pocket that could be probed, the CP sites showing PD ≥3 mm), and tooth mobility (TM, scored from 0 to 3, with 0 indicating no detectable movement) [16].

DNA microarray hybridization

P. gingivalis DNA was spotted on glass slides for hybridization with probes. Each DNA sample was spotted at least twice by a microarray robot (Micro-Grid II arrayer; BioRobotics Woburn, Mass., USA). *P. gingivalis* W83 genomic DNA was spotted five times on the slides as a positive control, and Arabidopsis DNA once on the slide as a negative control. DNA fragments isolated by suppression subtractive hybridization were labeled with Cy5-dUTP (Takara) through PCR amplification and used as probes. PCR amplification was in a 25-μl reaction volume with 0.4 μM primer with Cy5-dUTP. Thermal PCR conditions consisted of 30 cycles (30 s at 94°C, 30 s at 66°C, and 1 min 30 s at 72°C). Microarray slides were pre-hybridized at 60°C for 30 min in hybridization buffer containing 0.5 mg/ml denatured clupeine DNA. Probe mixtures were denatured at 95°C for 5 min and added to the pre-hybridized slides and sealed with a cover glass. Slides were hybridized in a HybChamber (Gene Machines, San Carlos, Calif., USA) at 80°C for 1 h followed by 65°C for 2 h and then washed for 10 min in solutions of 2 ×sodium chloride-sodium citrate (SSC), 0.2% sodium dodecylsulfate (SDS) followed by 0.1 ×SSC, 0.2% SDS, and finally in 0.1 ×SSC. Washed slides were dried at room temperature.

Scanning and microarray data analysis

Microarray slides were scanned at a resolution of 10 μm using a GenePix® 4000B microarray scanner (Axon instruments, Union City, Calif., USA). For consistent scanning of all hybridized slides, laser power and photomultiplier tube (PMT) gain were adjusted to 100%. The GenePix 4.0 image analysis software (Axon instruments) was used for spot intensity calculation. The signal-to-noise ratio (SNR) for each spot was calculated based on the following formula [17]: SNR = (signal intensity - background)/ standard deviation (SD) of background, in which the 'background' measurement refers to the local spot background intensity; the 'SD of background' was calculated across all pixels, as measured by the GenePix® software. When the signal-to-noise ratio (S/N) was ≥1.5, or when the median pixel intensity was ≥1000, a spot was scored as positive. The criteria for identifying a positive gene was set as follows: 1) if the two spots were both positive, the result was considered positive (+); 2) if one or no spot was positive, the result was considered negative (–).

Statistical analysis

The fluorescence intensities of Cy5 were analyzed. The intensities of fluorescent signals represented the quantities of tagged probes. Each test was repeated five times for each genechip and the results were analyzed. Fisher's exact test and Spearman correlation analysis were applied in this study (SPSS software, v. 13.0; SPSS, Chicago, Ill., USA). $P < 0.05$ was considered statistically significant and $p < 0.01$ as highly statistically significant.

Results

Bacterial strains and culture conditions

Clinical isolates of *P. gingivalis* were identified by Gram-staining and morphological examination, biochemical reaction and *P. gingivalis* specific 16S rDNA gene detection by PCR. Thirty strains of *P. gingivalis* were cultured and detected in 73% of Chinese CP patients. Twenty strains of *P. gingivalis*

were cultured and detected in 26% of periodontally healthy individuals. We selected 20 clinical isolates of *P. gingivalis* from disease regions as the study group and 20 clinical isolates of *P. gingivalis* from healthy regions as the control group. The two groups were similar in age and sex.

Identification of genes unique to P. gingivalis W83

Subtraction products were obtained from two rounds of subtractive hybridization and PCR amplification (Figure 1). These PCR fragments were subsequently cloned and sequenced. Among 194 clones obtained, 165 that had products from 100 to 500 bp were chosen for sequencing, and 18 unique genes were identified (Table I). These genes encompass a wide spectrum of putative functions and unknown functions. To confirm that they were indeed present in strain W83 but absent in strain ATCC33277, dot blot analysis was performed. The results showed that all 18 genes probed hybridized to chromosomal DNA from strain W83, and none hybridized to strain ATCC 33277.

Survey of clinical isolates for the presence of the W83 specific genes

To see whether there was any correlation between the presence of the strain W83 specific genes and the clinical isolates from diseased and normal sites, the 18 genes were used as probes in a DNA microarray assay to analyze DNA samples prepared from *P. gingivalis* clinical isolates. A total of 40 *P. gingivalis* isolates were analyzed, which represented samples obtained from 20 healthy and 20 periodontitis patients. As indicated in Table II, 10 of the 18 genes showed statistically significant correlation with *P. gingivalis* strains isolated from periodontitis patients ($p < 0.05$) (Figure 2).

To further verify the microarray results, 9 of the 18 genes were randomly selected to be amplified by PCR from the clinical samples. Of the 9 genes, 8 produced similar patterns as in the microarray, and 1 showed slightly different results. Fisher's exact test for PG0717 was $p < 0.01$ in the PCR analysis, but $p < 0.05$ in the microarray analysis.

Discussion

Periodontal diseases are among the most common infections of humans, with an estimated 5–20% of the world's population suffering from generalized periodontitis [18]. The development of periodontal disease is a multifactorial process involving interactions between the host and microorganisms that colonize the gingival sulcus. The Gram-negative anaerobe *P. gingivalis* has been strongly implicated in the initiation and progression of periodontal disease and possesses a sophisticated array of virulence factors [19]. The identification of genes that contribute to virulence in pathogenic strains of bacteria is a powerful tool in the study of microbial pathogenesis. This tool has often relied on determination of the phenotypic properties, biochemical activities, and immunological characteristics of pathogenic bacteria. Recently, these assays have been augmented by genomic DNA-based analyses that can identify species, strains, and even mutants within strains. The availability of complete genome sequences for many pathogenic bacteria has further increased the accuracy and specificity of such tests. In this study, we used SSH to identify genes

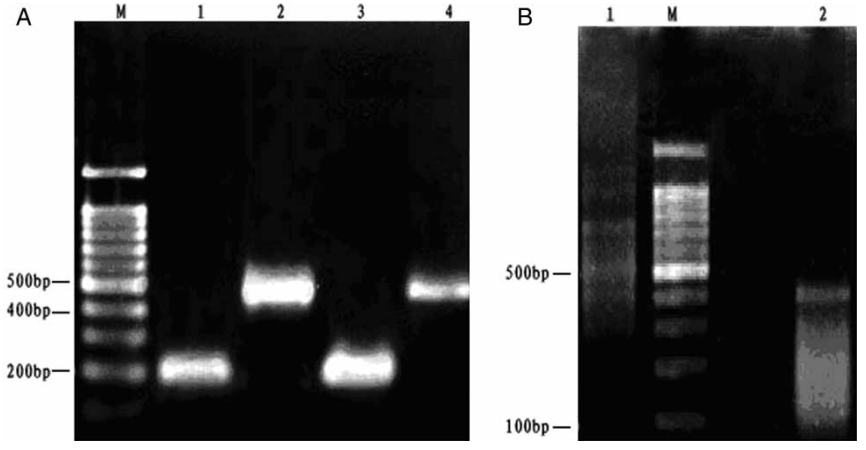

Figure 1. SSH product analysis. A. Results of ligation efficiency analysis showing that at least 25% of the tester DNA fragments have adaptors at both ends. M: 100 bp DNA Ladder: lane1: PCR products obtained using Tester 1-1 (Adaptor 1-ligated) as template with *P. gingivalis* 16S rRNA forward and reverse primer: lane 2: PCR products obtained using Tester 1-1 as template with *P. gingivalis* 16S rRNA forward primers and PCR primer 1; lane 3: PCR products obtained using Tester 1-2 (Adaptor 2R-ligated) as template and *P. gingivalis* 16S rRNA reverse and forward primers: lane 4: PCR products obtained using tester 1-2 as template and *P. gingivalis* 16S rRNA forward primer 1. B. PCR products obtained from subtracted DNA showing that tester-specific DNA was selectively amplified during the reactions. M: 100 bp DNA ladder; lane 1: Primary PCR products appear as a smear from 0.2–2 kb, with some distinct bands; lane 2: a secondary PCR amplification is performed using nested primers to further reduce background PCR products and to enrich for tester-specific sequences. Secondary PCR products appear as smears with a number of distinct bands.

Table I. Genes that are present in strain W83 but absent in strain ATCC 33277.

	Fragment length (bp)	Gene no.[a]	Encoded proteins[a]	Genbank no.[a]	E-value[b]
1	199 bp	PG0116	Conserved hypothetical protein	AE017172	5e–108
2	291 bp	PG0183	Lipoprotein, putative	AE017172	5e–163
3	197 bp	PG0544	Type I restriction-modification system, M subunit, putative	AE017173	4e–107
4	196 bp	PG0717	Lipoprotein, putative	AE017174	2e–106
5	215 bp	PG0742	Antigen PgaA	AE017174	8e–118
6	212 bp	PG0836	543bp at 5'side: hypothetical protein 1006 bp at 3'side: integrase	AE017174	1e–113
7	341 bp	PG0838	Integrase	AE017174	0.0
8	347 bp	PG0839	Conserved hypothetical protein	AE017174	0.0
9	238 bp	PG0861	Helicase, SNF2/RAD54 family	AE017175	2e–131
10	238 bp	PG1055	Thiol protease	AE017175	4e–129
11	184 bp	PG1059	Hypothetical protein	AE017175	2e–99
12	146 bp	PG1134	Thioredoxin reductase	AE017175	7e–77
13	152 bp	PG1436	ATPase, putative	AE017177	2e–80
14	192 bp	PG1503	LytB-related protein	AE017177	e–104
15	158 bp	PG1529	Hypothetical protein	AE0171771	e–81
16	238 bp	PG2100	Immunoreactive 63 KDa antigen PG102	AE017179.	e–122
17	116 bp	PG2135	Lipoprotein, putative	AE017179	1e–58
18	170 bp	PG2152	DNA-binding protein, histone-like family	AE017179	4e–91

[a]GenBank homology search results.
[b]The E-value is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size.

harbored only in virulent strains of *P. gingivalis*. Strains W83 (virulent) and ATCC 33277 (avirulent) were selected because previous reports have compared their virulence-associated activities and disease-promoting characteristics *in vitro* and *in vivo* [20–22]. The results suggested that these genes encode proteins whose function may contribute to pathogenicity. Putative functions of these genes are discussed below.

PG0836 and PG0838 encode putative transposase and integrases, respectively. These genes are often associated with transposable elements and acquisition of pathogenic island [23]. PG1055 encodes a putative thiol protease. This group of proteases is required for growth and other housekeeping functions, including processing enzymes for various cell surface proteins [24]. Although the primary function of proteases secreted by asaccharolytic bacteria such as *P. gingivalis* is to provide peptides for growth, they are also directly involved in tissue invasion and destruction, as well as evasion and modulation of host immune defenses [25]. PG0116 encodes a

Table II. Fisher's exact test of microarray hybridization results of *P. gingivalis* isolated from healthy and diseased sites.

No.	Length (bp)	Genes[a]	Disease region positive no.	Healthy region positive no.	*p*
1	199	PG0116	19	13	0.044[b]
2	291	PG0183	14	5	0.010[b]
3	197	PG0544	16	12	0.301
4	196	PG0717	18	11	0.031[b]
5	215	PG0742	16	8	0.022[b]
6	212	PG0836	14	3	0.001[c]
7	341	PG0838	16	7	0.010[b]
8	347	PG0839	16	8	0.022[b]
9	238	PG0861	13	7	0.113
10	238	PG1055	19	10	0.003[c]
11	184	PG1059	16	19	0.342
12	146	PG1134	12	8	0.343
13	152	PG1436	15	9	0.105
14	192	PG1503	15	9	0.105
15	158	PG1529	15	12	0.501
16	238	PG2100	16	6	0.004[c]
17	116	PG2135	14	6	0.026[b]
18	170	PG2152	16	14	0.716

[a]GenBank ID number.
[b]$P < 0.05$: statistically significant.
[c]$P < 0.01$: highly statistically significant.

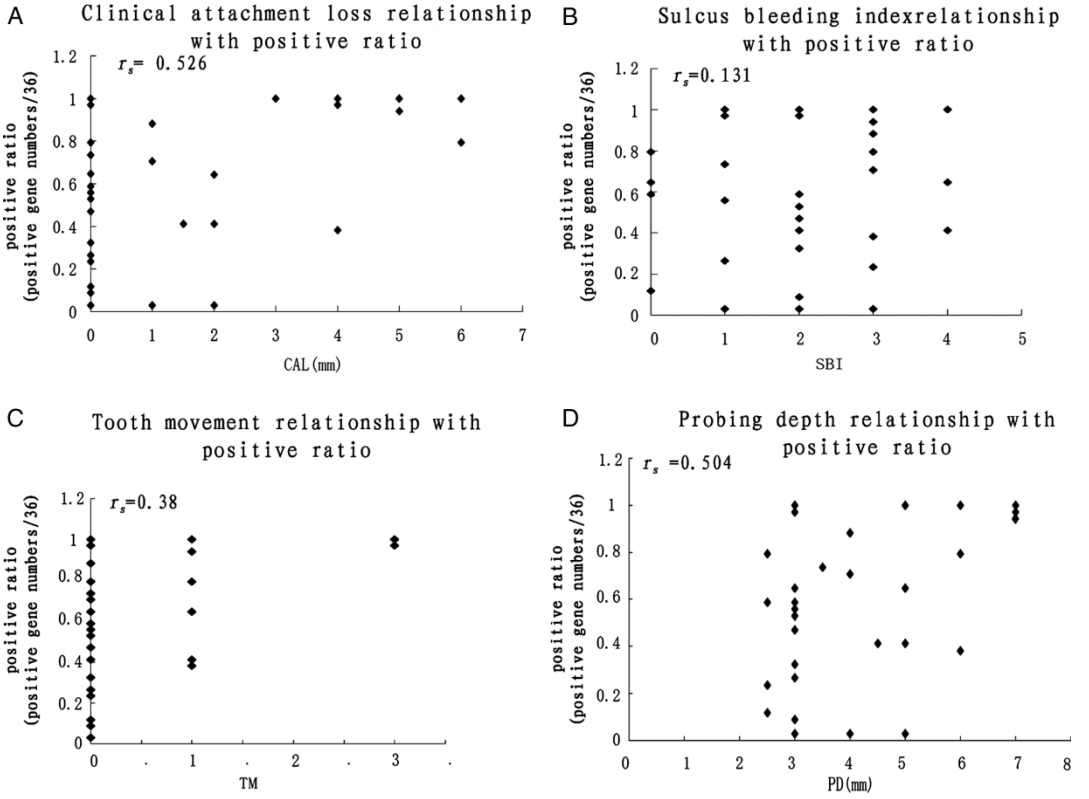

Figure 2. Scatter plot of Spearman correlation analysis results. A. X-axis: Clinical attachment loss (CAL). Y-axis: Positive ratio of *P. gingivalis* DNA was hybridized with probes. Spearman correlation analysis suggests that r_s value of CAL was 0.526, $p<0.01$. B. X-axis: Sulcus bleeding index (SBI) Y-axis: Positive ratio of *P. gingivalis* DNA was hybridized with probes. Spearman correlation analysis suggests that r_s value of SBI was 0.131, $p>0.05$. C. X-axis: Tooth mobility (TM) Y-axis: Positive ratio of *P. gingivalis* DNA was hybridized with probes. Spearman correlation analysis suggests that r_s value of TM was 0.38, $p<0.05$. D. X-axis: Probing depth (PD). Y-axis: Positive ratio of *P. gingivalis* DNA was hybridized with probes. Spearman correlation analysis suggests that r_s value of PD was 0.504, $p<0.01$.

conserved hypothetical protein, which may be involved in the synthesis of capsular polysaccharide. The severity of disease is correlated with the presence of the capsule and with the capsule serotype in a mouse infection model. The capsule of strain W83 (K1 type) is associated with the most severe form of infection, while strain 381, which does not possess a capsule (K^-), causes minimal infection [21]. PG0717, PG0183, and PG2135 encode lipoprotein. Several bacterial lipoproteins have been identified and suggested to play an important role in bacterial pathogenesis [26]. Lipoproteins also exhibit many biological activities associated with LPS [27]. PG0742 encodes PgaA, a cell-surface antigen that reacts with monoclonal antibody (mAb), LDS28, generated against cell-surface antigen of *P. gingivalis* [28]. PG2100 encodes immunoreactive 63 KDa antigen PG102. The function of these genes in pathogenesis is not known.

As an opportunistic pathogen, *P. gingivalis* often exists as a member of the oral indigenous flora, casing diseases only when the ecological balance is shifted. As reported, *P. gingivalis* was detected in 79% of subjects with periodontitis and in only 25% of periodontal healthy subjects [29]. In the subgingival area, bacteria experience dramatic environmental changes as a consequence of host eating and oral hygiene patterns, gingival crevicular flow rate variability, and degree of bleeding. In response to these dynamic processes, bacteria often regulate gene expression to maintain optimal phenotypic properties. Expression of virulence factors in a wide range of bacteria is tightly regulated in response to environmental cues [30]. Previous human studies have shown that the level of *P. gingivalis* in subgingival plaque has been associated with the severity of periodontitis as measured by periodontal pocket depth and loss of clinical attachment [31,32]. Ten genes are significantly associated with periodontitis ($p<0.05$ Table II and Figure 2). This may suggest that increasing CAL and deep periodontal pockets might promote growth of Gram-negative oral anaerobes, and might speed up the destruction of periodontal tissue.

In conclusion, using SSH we identified 18 genes that are present in the virulent strain W83 but absent in the avirulent strain ATCC 33277. Of these genes, 10 are significantly associated with periodontitis. This study provides further evidence that biodiversity exists among clinical isolates of *P. gingivalis* from healthy and diseased periodontal sites. Further studies are needed if we are fully to understand the function of these genes in pathogenesis.

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