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Identification of differentially expressed genes using an annealing control primer system in periodontitis

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In the gingival tissues of patients with periodontitis, inflammatory responses are mediated by a wide variety of genes. In this study, we screened for differentially expressed genes (DEGs) in periodontitis compared with normal tissue using an annealing control primer (ACP) system. By ACP RT-PCR analysis, we obtained about 160 amplicons, 8 of which were found to be differentially expressed. DEGs in patients with periodontitis were thus successfully and reliably identified by the ACP-based RT PCR technique. The DEGs identified in the screen may also enhance our understanding of the pathogenesis of periodontitis.

KEY WORDS: periodontitis ACP-PCR, differentially expressed genes

INTRODUCTION

Periodontal disease consists of a group of infections that leads to the inflammation of gingival tissues and destruction of periodontal tissues and, in severe cases, is accompanied by the loss of alveolar bonewith eventual teeth lose [1]. Mechanisms of host response in the periodontal tissues are complex and involve numerous systems of interactions. The interaction of bacterial antigen with the host immune system is believed to be the basis for the destructive inflammatory response found in periodontal disease. Specific pathogens such as *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggreagtibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) associated with plaque biofilm have been known to induce strong systemic and mucosal immune responses in the oral cavity [2,3]. Therefore, in the gingival tissues of patients with periodontitis, a wide variety of genes are either up- or down-regulated to mediate the inflammatory response.

Among several reverse transcription-polymerase chain reaction (RT-PCR) methods, differential display methods, which are based on PCR using short arbitrary primers, are simple and fast but produce high rates of false positives. Annealing control primer (ACP) system is a highly accurate and sensitive PCR technology that can be used to amplify the minute amounts of material available in tissues [4,5]. This primer anneals specifically to the template and allows only genuine product to be amplified, thus eliminating false-positives. In this study, differentially expressed genes (DEGs) in a normal gingiva vs. in an inflammatory gingiva were identified by using an ACP. In addition, the sequences of DEGs were analyzed.

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MATERIALS AND METHODS

Tissue sampling

Patients from Pusan National University Dental Hospital

with and without adult periodontitis were used as donor. Criteria for patient selection were: (1) absence of systematic diseases, (2) no antibiotics taken for at least 6 months before sampling, and (3) non-smokers. Informed consent was obtained from all donors and their rights were protected according to the protocol reviewed and approved by the institutional review board of Pusan National University Dental Hospital. Sites with no overt signs of gingival inflammation and with a probing depth of < 3mm were defined as clinically healthy; sites with obvious alveolar bone loss detected radiographically and a probing depth > 3were defined as exhibiting signs of periodontitis. Gingival samples were collected from both normal and periodontitis sites after infiltration with an appropriate local anesthetic. Samples comprised the epithelial lining and a portion of the underlying connective tissue. The gingival tissue specimens obtained were thoroughly rinsed with sterile normal saline solution, transferred into Eppendorf tubes, and stored -70℃ until use. The histolopathological diagnoses were determined and one sample from each group was used in the experiment.

RNA isolation and cDNA synthesis

Total RNA was isolated from both normal gingiva and inflammatory gingiva with the Trizol reagent (Invitrogen, Carlsbad, CA). Further purification of the extracted RNA was achieved by the use of the RNeasy total RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified RNA was quantified spectrophotometrically.

Reverse transcription was performed for 1.5 hr at 42℃ in a final reaction volume of 20 μl containing the purified mRNA, 4 ml of 5 × reaction buffer (Promega, Madison, WI), 5 ml of dNTP (each 2mM), 2 ml of 10 mM cDNA synthesis primer dT-ACP1 (Table 1), 0.5 ml of RNasin

Table 1. List of primers used in ACP-based PCR

Primer name	Sequnece
cDNA synthesis primer	
dT-ACP1	5'-CTGTGAATGCTGCGACTACGATIIII(T)18-3'
Reverse primer	
dT-ACP2	5'-CTGTGAATGCTGCTGCGACTACGATIIIII(T)15-3'
	Arbitrary primer (forward primer)
ACP1	5'-GTCTACCAGGCATTCGCTTCATIIIIIGCCATCGACC-3'
ACP2	5'-GTCTACCAGGCATTCGCTTCATIIIIIAGGCGATGCC-3'
ACP3	5'-GTCTACCAGGCATTCGCTTCATIIIIICCGGAGGATG-3'
ACP4	5'-GTCTACCAGGCATTCGCTTCATIIIIIGCTGCTCGCG-3'
ACP5	5'-GTCTACCAGGCATTCGCTTCATIIIIIAGTGCGCTCG-3'
ACP6	5'-GTCTACCAGGCATTCGCTTCATIIIIIGGCCACATCG-3'
ACP7	5'-GTCTACCAGGCATTCGCTTCATIIIIICTGCGGATCG-3'
ACP8	5'-GTCTACCAGGCATTCGCTTCATIIIIIGGTCACGGAG-3'
ACP9	5'-GTCTACCAGGCATTCGCTTCATIIIIIGATGCCGCTG-3'
ACP10	5'-GTCTACCAGGCATTCGCTTCATIIIIITGGTCGTGCC-3'
ACP11	5'-GTCTACCAGGCATTCGCTTCATIIIIICTGCAGGACC-3'
ACP12	5'-GTCTACCAGGCATTCGCTTCATIIIIIACCGTGGACG-3'
ACP13	5'-GTCTACCAGGCATTCGCTTCATIIIIIGCTTCACCGC-3'
ACP14	5'-GTCTACCAGGCATTCGCTTCATIIIIIGCAAGTCGGC-3'
ACP15	5'-GTCTACCAGGCATTCGCTTCATIIIIICCACCGTGTG-3'
ACP16	5'-GTCTACCAGGCATTCGCTTCATIIIIIGTCGACGGTG-3'
ACP17	5'-GTCTACCAGGCATTCGCTTCATIIIIICAAGCCCACG-3'
ACP18	5'-GTCTACCAGGCATTCGCTTCATIIIIICGGAGCATCC-3'
ACP19	5'-GTCTACCAGGCATTCGCTTCATIIIIICTCTGCGAGC-3'
ACP20	5'-GTCTACCAGGCATTCGCTTCATIIIIIGACGTTGGCG-3'

Plus RNase Inhibitor (Promega, Madison, WI) and 1 ml of Superscript II reverse transcriptase (200 U/μl) (Invitrogen, Carlsbad, CA). First-strand cDNAs were diluted with 120 ml of sterile water.

ACP-based PCR

The cDNAs are subjected to second-strand cDNA synthesis by random PCR amplification using dT-ACP2 and one of 20 arbitrary ACPs (Table 1). Second-strand cDNA synthesis was carried out at 50℃ during one cycle of first-stage PCR in a final reaction volume of 49.5 ml containing 1 ml of the diluted first-strand cDNA, 5 ml of 10 × PCR reaction buffer (Roche Applied Science, Mannheim, Germany), 5 ml of dNTP (each 2mM), 1 ml of 10 mM dT-ACP2, and 1 ml of 10 mM arbitrary primer. The tube containing the reaction mixture was held at 94℃ while 0.5 ml of Taq DNA polymerase (5U/ul) (Roche Applied Science, Mannheim, Germany) was added to the reaction mixture. The PCR protocol for second-strand synthesis was one cycle at 94℃ for 1 min, followed by 50℃ for 3 min, and 72℃ for 1 min. After second-strand DNA synthesis was completed, 40 amplification cycles wereperformed. Each cycle involved denaturation at 94℃for 40 sec, annealing at 65℃ for 40 sec, and extension at 72℃was performed to complete the PCR. The amplified PCR products were then subjected to electrophoresis on 2% agarose gels and stained with 1 mg/ml ethidium bromide.

Identification of differentially expressed genes

The differentially expressed bands were extracted and cloned into a TOPO vector of the TA cloning kit (Invitrogen, Carlsbad, CA) and used to transform competent TOP10 Escherichia coli cells. The colonies were grown for 18 hrs at 37℃ on Luria broth agar plates containing ampicillin, X-gal (5-bromo 4-chloro 3-indoyl-β-D-galactopyranoside), and isopropyl- β-D-thiogalactopyranoside for blue/white colony selection. In order to verify the identity of inserted DNA, isolated plasmids were sequenced automatically (Applied Biosystems, Foster city, CA). Complete sequences were analyzed by searching for similarities using BLASTN or BLASTX search program at the National Center for Biotechnology Information (NCBI) GenBank.

RESULTS

To identify genes which were differentially expressed in gingiva from healthy and periodontitis, the mRNAs from both types of gingiva were extracted and subjected to ACP RT-PCR analysis using a combination of 20 arbitrary primers and two anchored oligo(dT) primers (dT-ACP 1 and dT-ACP 2). The analysis produced about 160 amplicons, 8 of which were differentially expressed (Fig. 1). We have found 7 genes that were markedly up-regulated in a gingivafrom patient with periodontitis, while only one was enhanced in a healthy gingiva. The sequence of a total 8 DEGs analyzed by BLAST search program were determined (Table 2). One DEG up-regulated in a normal gingiva was for the constant region of Homo sapiens immunoglobulin kappa chain, while the expressions of two DEGs coding for the variable region of kappa chain and lamda constant were enhanced in an inflammatory gingiva. Other DEGs increased in an inflammatory gingiva were for nuclear ribonucleoprotein, hydroxyacyl glutathione hydrolase, and plasminogen activator inhibitor-1 mRNA-binding protein (PAI-RBP1).

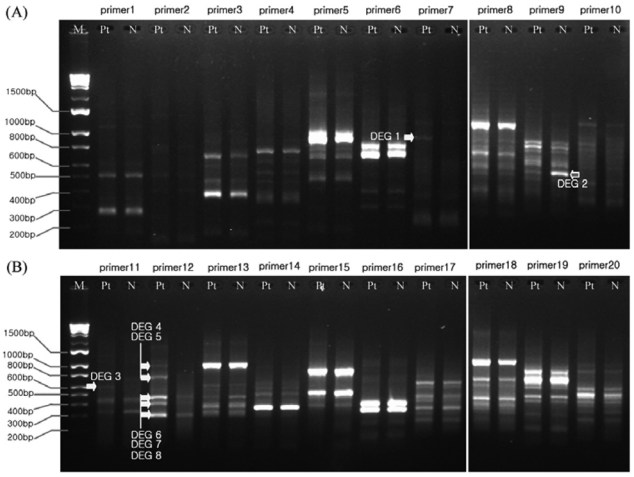

Fig. 1. Differentially expressed genes in gingiva from healthy and periodontitis patient. Band patterns for differential expression between a normal gingiva and a inflammatory gingiva after annealing control primer-polymerase chain reaction (ACP-PCR) using ACP1 to ACP10 (A) and ACP 11 to ACP 20 (B). Total RNA was isolated from both tissues and subjected to reverse transcription-polymerase chain reaction (RT-PCR) using ACP. The PCR products were separated by electrophoresis on 2% agarose gels. M, lane containing a 100-bp DNA ladder.

DISCUSSION

In periodontal tissues, a number of genes are up- or

Table 2. List of genes identified by ACP system

DEG No.	GenBank Accession No.	Sequence homology search
DEG 1	BC036082	Homo sapiens,Similar to HSPC159 protein, clone IMAGE : 5301908, mRNA
DEG 2	BC090336 (BlastN)	Homo sapiens immunoglobulin kappa constant, mRNA (cDNA clone MGC : 88341 IMAGE : 30330282), complete cds
	BAC01760 (BlastX)	Immunoglobulin kappa light chain VLJ region [Homo sapiens].
	CR609605 (BlastN)	Full-length cDNA clone CS0CAP007YC20 of thymus of Homo sapiens (human).
DEG 3	BC073792 (BlastN)	Homo sapiens immunoglobulin kappa variable 1-5, mRNA (cDNA clone MGC : 88810 IMAGE : 6280971), complete cds
	BAC01738 (BlastX)	immunoglobulin kappa light chain VLJ region [Homo sapiens].
DEG 4	AK129814 (BlastN)	Homo sapiens cDNA FLJ26303 fis, clone DMC07819, highly similar to HETEROGENEOUS NUCLEAR REBONUCLEOPROTEIN A1
	AAH52296 (BlastX)	Heterogeneous nuclear ribonucleoprotein A1, isoform
DEG 5	NM_15640	Homo sapiens PAI-1 mRNA-binding protein (PAI-RBP1), mRNA
DEG 6	HS429E7 (BlastN)	Human DNA sequence from clone LA16-429E7 on chromosome 16 Contains the HAGH (hydroxyacyl glutathione hydrolase) gene, ESTs, STSs, GSSs and CpG Islands, complete sequence
	NM_005326 (BlastN)	Homo sapiens hydroxyacylglutathione Hydrolase (HAGH), mRNA
	NP_005317 (BlastX)	Hydroxyacyl glutathione hydrolase [Homo sapiens].
DEG 7	BC034142	Homo sapiens immunoglobulin kappa variable 1-5, mRNA (cDNA clone MGC : 32715 IMAGE : 4694346), complete cds
DEG 8	BC071804	Homo sapiens immunoglobulin lambda constant 2 (Kern-Oz-marker), mRNA (cDNA clone MGC : 88451 IMAGE : 4717660), complete cds

down-regulated by subgingival plaque biofilm comprised of facultative or anaerobic bacteria such as P. gingivalis, *Tannerella forsythia* and *A. actinomycetemcomitans* [1]. The primary mediators, such as IL-1b and TNF-αhave been shown to contribute to several events that are essential for the inflammatory response. Moreover, various inflammatory mediators including cytokines, chemokines and their receptors are expressed by gingival fibroblast, epithelial cells, endothelial cells and inflammatory cells in periodontally disease tissues [6-8]. In this study, we have searched for the genes expressed differentially in a gingiva from periodontitis patients compared to a healthy gingiva.

We used the ACP system to search DEGs in gingival tissues. The ACP system is based on two principles: the unique tripartite structure of the primers, which have distinct 3'- and 5'- end regions that are separated by a polydeoxyinosine [poly(dI)] linker, and the interaction of each region during two-stage PCR [4,5]. For first-strand cDNA synthesis, the reaction was carried out with dT-ACP1, wherein the 3'- end core portion comprises a hybridizing sequence complementary to a poly A region of mRNA transcripts. Polymerase chain reaction was conducted by using arbitrary ACPs to synthesize second-strand cDNAs under annealing conditions such that the 3'-end core portion of the dT-ACP2 is prevented from annealing to first-strand cDNAs and only the 3'-end core portion of the arbitrary ACP anneals to the first-strand cDNA. Arbitrary ACPs contain random sequence 10-mers as the 3'- end core sequences, and only those ACPs that are sufficiently complementary to a region of a first-strand cDNA anneal. Thus, the ACP system allows only real products to be amplified and eliminates false-positive results.

In present study, using ACP system, 8 DEGs were detected (Fig 1, Table 2). Seven DEGs including the variable region of kappa chain, nuclear ribonucleoprotein, hydroxyacyl glutathione hydrolase, and PAI-RBP1 were identified in an inflammatory gingiva. Among them, the increased expression of PAI-RBP1 is especially noticeable. PAI-RBP1 has been reported to be increased in non-small cell lung cancer with different tumor metastatic potential and ovarian cancer associated with tumor progression [9,10]. PAI-RBP1 has been suggested to be involved in tumor

development orinvasion and metastasis that are mediated via angiogenesis and cell migration. PAI-RBP1 is a binding protein for plasminogen activator inhibitor-1 (PAI-1). A major role of PAI-1 in immune response is the regulation of cell migration, thereby suggesting that PAI-1 may also modulate cellular recruitment during the acute inflammatory process [11-13]. PAI-1 expression is regulated by growth factors, cytokines, and hormones, including agents that regulate cellular cAMP levels [14-16].

The initialstages of periodontal inflammation are accompanied by vascular hyperpermeability and LPS from *Porphyromonas gingivalis* has been reported to induce vascular permeability [17]. PAI-1 can mediate vascular permeability [18] suggesting the possibility of regulating host response in periodontal disease. In relation to periodontal disease, it was reported that there was a significant increase of plasminogen activator activity-2and t-PA antigen in samples from inflamed gingival tissue compared with normal gingival tissue [19-21]. It has been suggested that t–PA and PAI-2 may play a significant role in the periodontal tissue destruction and tissue remodeling because of the increased concentration of t–PA and PAI-2 in inflamed gingiva and gingival crevicular fluid [22-24]. Deppe et al. reported that urokinase-type plasminogen and its inhibitor PAI-1 antigen was detected in normal or inflamed periodontal tissues [25].

In this study, 8 DEGs were identified in a normal gingiva vs. in an inflammatory gingiva using 20 ACPs. However, inflammatory cytokines and chemokines that are frequently reported in inflammatory diseases were not detected in this study, which limit our study. It is expected that more DEGs will be detected if more ACPs will be used. In future, further studies will be needed to detect more new DEGs serving as the targets for control or therapy of periodontal disease and to elucidate the relation between PAI-1 or PAI-RBP1 and periodontitis.

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