行政院國家科學委員會專題研究計畫　成果報告

國人 FMLP 受器多形性的分佈及作為不同類型牙周炎危險因子之可行性

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前言:

Periodontitis is an infectious disease mainly caused by Gram negative anaerobic bacteria. It is the imbalance between destructive versus defensive responses of host immune system to these periopathogenic microbes, which finally cause pocket formation, attachment loss and alveolar bone loss. However, the severity of periodontal disease can not be simply explained by the quantity and composition of periodontal related dental plaque. In addition to the periopathogenic microbes, other possible factors involved in the severity include smoking, tooth morphology, systemic diseases and genetics, etc.; associated evidence of genetic factors in periodontal disease have be shown in early onset periodontitis.

研究目的:

This study was limited to look at the host defensive immunity, focusing on the relationship between the susceptibility to generalized aggressive periodontitis (GAgP formerly GJP, G-EOP) and chronic periodontitis (CP, formerly AP) and genetic polymorphisms of FMLP receptor at PMN surface.

文獻探討:

When neutrophil functions are concerned by periodontists, depressed phagocytosis and chemotaxis are well documented in early onset periodontitis for years.[1,2,4,5,20] Evidence already shows defective neutrophil chemotaxis in early onset periodontitis. This alteration may occur at complement C5a,[1(localized juvenile periodontitis, LJP), 2(G-EOP, LJP), 3(LJP)] GP-110[1(LJP) ,4(LJP, G-EOP)] and FMLP[1(LJP), 2(G-EOP, LJP), 4(LJP, G-EOP), 5(G-EOP, LJP), 6(G-EOP, LJP)] receptors. The PMN chemotactic response includes chemoattractant recognition, transmembrane signaling transmission, cytoskeletal movement and cell motility, and initiated by the binding of FMLP and specific receptor at PMN plasma membrane. The chemotaxis can be stimulated by various N-formylated peptides such as N-formyl-1-methionyl-1-leucyl-1-phenylalanine (FMLP) which are thought structural analogs of bacterial products involved in the neutrophil chemotaxis to bacterial infection. Studies have shown that the binding of FMLP by PMN from LJP patients is reduced by 50% compared to that of cells from normal controls.[7] It is also pointed that this difference is not quantitative but qualitative alteration in the nature of FMLP receptors in LJP.[8,9] Although controversy exists between studies, FMLP receptors from LJP and control subjects do show different binding pattern with ligands such as anti-receptor monoclonal antibodies.[8] Another interesting study reported the similar PMN migration and adhesion between G-EOP and control subjects, however, the superoxide production was significantly reduced when GJP PMNs were stimulated by FMLP[10], **w**hich suggesting a dysregulation of the signal transduction pathway distal

to FMLP receptors.

FMLP receptor is a cell-surface protein, G-protein coupled glycoprotein and is encoded primarily by a 1.6Kb mRNA and 7.5Kb structural gene located on chromosome19q13.3.[11,12] Its coding region is on exon-2 (about 1 Kb)[13] after exon-1 and a 5 Kb intron region. The first extracellular loop closet to the N-terminal end of the FMLP receptor exhibits the strongest ligand binding compared to other loop structures, and residue Arg-84 and Lys-85 on this loop play major roles in ligand-binding activity which is not thought a structure-dependent but a charged moiety dependent nature.[14]

Two polymorphisms in the second intracellular loop (the 3rd transmembrane helix) of FMLP receptors have been reported at base 329 T to C and base 378 C to G. The mutation at base 329 alters a phenylalaine to serine at residue 110 which shifts hydrophobic residue to a polar amino acid and more hydrophilic; the change at base 378 results to a shift from cysteine with a sulfur group to tryptophan (hydrophobic) with a large function group at residue 126. These two changes are all located at the contact site of the G-protein receptor[15] and may irreversibly alter the structure of this receptor and its following binding ability.[16,17] The neutrophils from LagP (localized aggressive periodontitis) patients demonstrated a significant reduction in chemotaxis toward FMLP compared to those of chronic periodontitis patients and controls.[6] This finding was correlated with the low surface expression of FPR or chemotaxis toward FMLP at the change of 110; also with low G-protein coupling and chemotaxis at the change of 126.[17] The 110 change was considered more defective than 126 change.[17,18] A recent study[27] reported there was statistic significance (P=0.0005) about the changes in base 329 T to C and base 378 C to G from adult periodontitis and healthy controls to localized juvenile periodontitis (LJP/LagP). When different races were examined, the base alterations was also highly related to African-American than Caucasian (P=0.005)[19] and this finding might partially explain the high frequency of LJP in African-American. In adult periodontitis, the presentation of such polymorphism was not seen which suggested its specificity to LJP cases. However, there is no data available in generalized aggressive periodontitis (GAgP) in any ethnical population.

研究方法:

1. Selection of subjects

Systemically healthy subjects were recruited from the outpatient group or staff members in the dental clinic of a local hospital; all subjects provided written informed

consent. Subjects with a history of diabetes, smoking, HIV infection, anti-inflammatory drug or antibiotic use within the previous 6 months, pregnancy, need for antibiotic prophylaxis, and receiving any periodontal treatment were excluded. The clinical periodontal examination included plaque index, probing pocket depth, clinical attachment level, bleeding on probing and radiographic evaluation. Only subjects retaining more than 18 teeth were included. Subjects were categorized into 3 groups according to the following criteria:

(1) Chronic periodontitis (age >35 years) with attachment loss≧2 mm on more than 1 tooth; more than 3 sites with probing pocket depth ≥6 mm; and lesions distributed on more than 2 teeth in each quadrant;
(2) Generalized aggressive periodontitis (age ≦35 years) with more than 8 teeth with AL ≧5 mm; probing pocket depth ≧6 mm; and at least 3 of these involved teeth were not first molars and incisors;
(3) Healthy controls who included subjects of any age with no evidence of attachment loss at more than 1 site or probing pocket depth ≧3 mm.

2. Isolation of genomic DNA

Genomic DNA was isolated from peripheral blood of each subject using a DNA isolation kit (Puregene, Gentra System, USA) according to manufacturer's instructions. Isolated DNA was stored at -20°C before further genotype analysis.

3. Determination of FMLP receptor genotypes

PCR amplification:

在冰上製備 PCR reaction mixture (Master Mix)：

FMLP: ASO (allele specific oligonucleotide) probes

(control, 329T/110F) ATC AAC TTG T**T**C GGA AGT GTC
(a.a. 110 phenalanine)

(patient, 329C/110S) ATC AAC TTG T**C**C GGA AGT GTC
(a.a. 110 serine)

(control, 378C/126C) GC TGT GTT TG**C** GTC CTG CAT C
(a.a. 126 cysteine)

(patient, 378G//126W) GC TGT GTT TG**G** GTC CTG CAT C
(a.a. 126 tryptophan)

Sense primer forward 5'CTC TCC CCA CGA ACA TCT CTG
Antisense primer reverse 5'CAA CGG CCA CAT TTA TCC TCT
PCR product: 570 bp fragment

Prepare the PCR reaction mixture (Master Mix) on ice

H_2O	16.9
10X Buffer, 100mMTris.HCl pH=8.3, 500 mM KCl	2.5
$MgCl_2$ 2.5mM	2
dNTP 2.5mM each	1
forward primer,10 pmole/μl	1
reverse primer,10 pmole/μl	1
AmpliTaq Gold 5U/μl (Perkin-Elmer®, Norwalk, CT, USA)	0.05
DNA solution 100ng/μl	0.5
Volume (μl)	25

FMLP 329T/C,378C/G：

	temperature℃	minute	second	Cycle	
1	94	10		1	Hot start
2	94 60 72	 1	30 30	30	Denature Annealing Extension
3	72	5		1	
4	4				soaking

*2% agarose TBE gel check the PCR product:8-10μl PCR product

Hybridization:製備標準的 standard hybridization buffer

5X SSC / 0.1%N-lauroylsarcosine / 0.02%SDS / 1% blocking reagent stock solution (herring/salmon sperm DNA)。

1) Put nylon membrane hybridzed with PCR product in hybridization glass tube covered by 15ml hybridization buffer for 30 min at 65℃ (block nonspecific binding with probe)。

2) 4 pieces of hybridzed nylons in labeled probe and cold probe for 4hr at 65℃

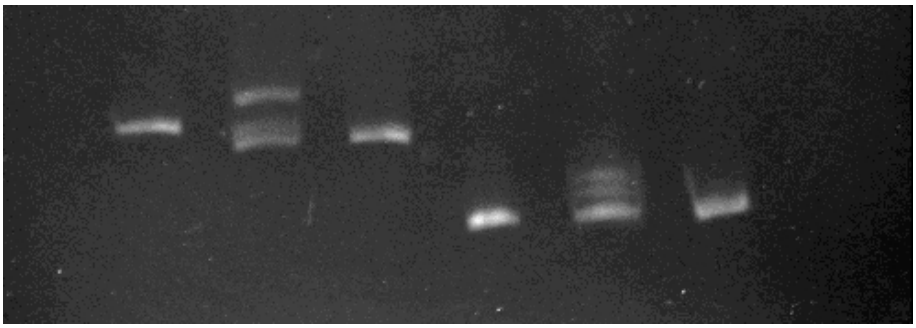
Dig-oligonucleotide 3'-End labeling kit to label the ASO probe

	110F	110S	126C	126W
10 μM hot	110F 2μl	110S 2μl	126C 2μl	126W 2μl
cold	110S 20μl	110F 20μl	126W 20μl	126C 20μl

The haplotypes of FMLP polymorphisms were also confirmed by TTGE (Temporal Temperature Gradient Electrophoresis).

The following picture was a representative of the result in our laboratory which only showed the high differentiation ability of TTGE in polymorphism study (pilot model study, not real samples).

1 2 3 4 5 6



1: 110F
2: 110F/110S mix
3: 110S
4: 126C
5: 126C/126W mix
6: 126W

結果與討論:

Distribution of FMLP receptor polymorphisms among different groups of subjects

	Genotypes	GAgP patients (n=26)	CP patients (n=49)	Healthy Controls (n=74)
110 F/S				
	F/F	26	49	74
	F/S	0	0	0
	S/S	0	0	0

	Genotypes	GAgP patients (n=28)	CP patients (n=49)	Healthy Controls (n=74)
126C/W				
	C/C	28	49	74
	C/W	0	0	0
	W/W	0	0	0

1. All subjects were tested homozygotes of F/F and C/C without any heterozygote or F/S and C/W heterozygote in this study.
2. There were no differences between these three groups associated with FMLP receptor polymorphism distribution.
3. No significant difference in the polymorphism distribution was found in either adult periodontitis (chronic periodontitis) or healthy control neither in this study which was in accordance with the reports of Gwinn et al.[19] However, only homozygotes of F/F and C/C in our Taiwanese population was considered quite unique.
4. We can only suggest that the FMLP receptor polymorphism is not associated with the etiology of generalized aggressive periodontitis in Taiwan, although it was reported strongly related to the localized aggressive periodontitis in African-Americans.
5. To further elucidate the dilemma, studies focused at the localized lesion in Taiwanese should be performed.

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