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ANTIVIRAL INNATE IMMUNE RESPONSE IN PERIODONTAL TISSUE : PART I DETECTION OF ANTIVIRAL PROTEINS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Periodontics

Department of Periodontology
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นริศรา วนวิทย์ : การตอบสนองทางระบบภูมิคุ้มกันโดยกำเนิดต้านต่อไวรัสในเนื้อเยื่อปริทันต์ : ตอนที่ 1 การตรวจหาโปรตีนต้านต่อไวรัส. (ANTIVIRAL INNATE IMMUNE RESPONSE IN PERIODONTAL TISSUE : PART I DETECTION OF ANTIVIRAL PROTEINS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. รังสินี มหานนท์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. สาธิต พิชญางกูร, 59 หน้า.

โรคปริทันต์อักเสบเป็นโรคที่เกี่ยวข้องกับการอักเสบเรื้อรังของอวัยวะปริทันต์ และอาจนำไปสู่การสูญเสียฟัน สาเหตุหลัก คือ แบคทีเรีย ข้อมูลในเร็วๆนี้บ่งถึงบทบาทของไวรัสในการเกิดและการดำเนิน โรคปริทันต์อักเสบ อย่างไรก็ตามความรู้เกี่ยวกับการตอบสนองทางระบบภูมิคุ้มกันโดยกำเนิดต้านต่อไวรัสในเนื้อเยื่อปริทันต์ยังคงมีน้อยมาก ดังนั้นการศึกษานี้จึงเป็นการศึกษาแรกในการตรวจหาการแสดงออกของ เมสเซนเจอร์อาร์เอนเอ (mRNA) ของโปรตีนต้านต่อไวรัส (antiviral protein) คือ ซีเครโทรี ลิวโคไซท์ โปรทีเอส อินหิบิเทอร์ (secretory leukocyte protease inhibitor,SLPI), โปรตีน ไคเนส อาร์ (protein kinase R, PKR), โอลิโกอะดีไนเลท ซินเทเตส (oligoadenylate synthetase, OAS) และมิกโซไวรัส รีซิสแทนซ์ เอ (myxovirus resistance A, MxA) ด้วยวิธีเรียลไทม์ รีเวอร์สทรานส์คริปชัน-โพลิเมอเรส เชน รีแอคชัน (real-time RT-PCR) พบว่ามีการแสดงออกของ mRNA ของ SLPI, PKR, OAS และ MxA ทั้งในเนื้อเยื่อที่เป็นโรคปริทันต์อักเสบและเนื้อเยื่อที่มีสุขภาพดี (กลุ่มละ 5 ตัวอย่าง) โดยไม่พบความแตกต่างระหว่างกลุ่มอย่างมีนัยสำคัญ เนื่องจาก MxA มีความเกี่ยวข้องกับการต้านต่ออาร์เอนเอ (RNA) และดีเอนเอ (DNA) ไวรัส เราจึงมุ่งเน้นไปที่การแสดงออกในระดับโปรตีนของ MxA ในเยื่อบุผิวเหงือก (gingival epithelium) ของผู้ป่วยที่เป็นโรคปริทันต์อักเสบ (จำนวน 7 ตัวอย่าง) เปรียบเทียบกับเนื้อเยื่อปริทันต์ที่มีสุขภาพดี (จำนวน 9 ตัวอย่าง) ด้วยการย้อมทางอิมมูโนฮิสโตเคมี (immunohistochemical staining) พบว่าการย้อมติดสีของโปรตีน MxA ในชั้นเยื่อบุผิวของเนื้อเยื่อปริทันต์ที่มีสุขภาพดีมีระดับสูงกว่าเนื้อเยื่อปริทันต์ที่เป็นโรคปริทันต์อักเสบ และที่น่าสนใจ คือ ในชิ้นเหงือกที่มีสุขภาพดีชิ้นหนึ่งที่มีองค์ประกอบครบทั้งเยื่อบุผิวช่องปาก (oral epithelium), เยื่อบุผิวร่องเหงือก (sulcular epithelium) และเยื่อบุผิวเชื่อมต่อ (junctional epithelium) พบว่าย้อมติดสี MxA เข้มมากบริเวณร่องเหงือกที่เป็นตำแหน่งที่อยู่ใกล้ชิดกับคราบจุลินทรีย์ ซึ่งประเด็นนี้ควรมีการศึกษาเพิ่มเติมต่อไป และเนื่องจากเป็นที่ทราบกันดีว่า MxA ถูกเหนี่ยวนำโดยอินเตอร์เฟอรอน ชนิดที่ 1 เราจึงตรวจหาการแสดงออกของไซโตไคน์ (cytokine) นี้ในเนื้อเยื่อ ปริทันต์ด้วยวิธี real-time RT-PCR แต่กลับพบว่าแทบจะไม่พบอินเตอร์เฟอรอน ชนิดที่ 1 ในเนื้อเยื่อปริทันต์เลย ซึ่งชี้ให้เห็นว่าน่าจะมีสารชนิดอื่นที่เกี่ยวข้องกับการเหนี่ยวนำการสร้าง MxA ในเนื้อเยื่อปริทันต์ที่มีสุขภาพดี โดยสรุป เป็นการศึกษาแรกที่พบการแสดงออกของ mRNA ของโปรตีนต้านต่อไวรัสหลายชนิด คือ SLPI, PKR, OAS และ MxA ในเนื้อเยื่อปริทันต์ทั้งที่เป็นและไม่เป็นโรค นอกจากนั้นการปรากฏของโปรตีนเหล่านี้ในเนื้อเยื่อที่มีสุขภาพดี โดยเฉพาะอย่างยิ่ง MxA ในร่องเหงือก ชี้ให้เห็นถึงระบบภูมิคุ้มกันโดยกำเนิดต้านต่อไวรัสที่มีประสิทธิภาพของช่องปาก ซึ่งกลไกการทำงานและบทบาทหน้าที่ของโปรตีนเหล่านี้จำเป็นต้องมีการศึกษาเพิ่มเติมต่อไป

ภาควิชา .....ปริทันตวิทยา..... ลายมือชื่อนิสิต.....นริศรา วนวิทย์.....

สาขาวิชา.....ปริทันตศาสตร์..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS: ANTIVIRAL PROTEIN / INNATE IMMUNITY / PERIODONTAL TISSUE

NARISARA VANAVIT: ANTIVIRAL INNATE IMMUNE RESPONSE IN PERIODONTAL TISSUE : PART I DETECTION OF ANTIVIRAL PROTEINS. THESIS ADVISOR: ASSOC. PROF. RANGSINI MAHANONDA, Ph.D, THESIS CO-ADVISOR: SATHIT PICHYANGKUL, Ph.D, 59 pp.

Periodontitis is a common chronic bacterial inflammatory disease in oral cavity. It affects tooth supporting structure-periodontium and may cause tooth loss. Several recent studies have documented a role of viruses in the development and progression of periodontitis but little that we know about the host immune response to viruses. In this study, we explored the innate antiviral proteins (secretory leukocyte protease inhibitor (SLPI), protein kinase R (PKR), oligoadenylate synthetase (OAS), and myxovirus resistance A (MxA)) in periodontal tissues, comparing between periodontitis and healthy (n=5 in each group). By real-time reverse transcription-polymerase chain reaction, we found expression of SLPI, PKR, OAS, and MxA in periodontitis as well as in healthy tissues and there were no significant differences between the two groups. MxA protein involves in antiviral activity against both RNA and DNA virus, we then focused on the protein expression of MxA in gingival epithelium and compare its expressions between periodontitis (n=7) and healthy (n=9). By immunostaining, healthy tissues showed a higher score of MxA in epithelial layer than those in periodontitis. Of particular interest, we could obtain one complete biopsy of the oral, sulcular and junctional epithelium from healthy tissue. Very strong MxA expression was observed in gingival sulcus area, the strategic location in close proximity to dental plaque biofilms. The significance of this finding needs to be explored. Since MxA is known to be induced by type I interferon, we further examined the presence of this cytokine in periodontal tissues by real-time reverse transcription-polymerase chain reaction. Interestingly, negligible expression of type I interferon was detected, indicating that other mediators or molecules may involve in MxA induction in healthy tissues. In conclusion, this study is the first to report detection of mRNA expression of variety of antiviral proteins (SLPI, PKR, OAS, and MxA) in both periodontitis and healthy tissues. Expression of these proteins in healthy periodontal tissues, especially MxA in gingival sulcus, suggesting effective antiviral innate immunity in the oral cavity. Future research is required for better understanding of the mechanisms and signaling pathway of these antiviral proteins in healthy and periodontitis.

Department.....Periodontology..... Student's signature.....

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LIST OF ABBREVIATIONS

ANUG	acute necrotizing ulcerative gingivitis
ASFV	African swine fever virus
bp	base pair
cDNA	complementary DNA
C_t	threshold cycle
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ds	double stranded
EBV	Epstein-Barr virus
eIF2	eukaryotic initiation factor 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCF	gingival crevicular fluid
GTPases	guanosine triphosphatases
HBDs	human β-defensins
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HNPs	human α-defensins , human neutrophil peptides
HPV	human papilloma virus
HRP	Horseradish peroxidase
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IgG	immunoglobulin G
IL-8	interleukin-8
ISGs	IFN-stimulated genes

JAK	Janus kinase
kDa	kiloDalton
LL-37	cathelicidin
mm	millimeter
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MxA	myxovirus resistance A
OAS	2',5'-oligoadenylate synthetase
OCT	Optimum Cutting Temperature
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PKR	protein kinase R
PMN	polymorphonuclear cell
QRT-PCR	real-time quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
SLPI	secretory leukocyte protease inhibitor
STAT	signal transducer and activator of transcription
TLRs	Toll-like receptors
VSV	vesicular stomatitis virus

CHAPTER I

INTRODUCTION

1.1 Background of present study

Periodontitis is a chronic bacterial infection that affects the gingiva, periodontal ligament, cementum and bone supporting the teeth. Bacterial plaque stimulates the host inflammatory response leading to tissue damage. Even though the etiologic importance of bacteria in periodontitis has been understood for decades, several recent studies have also documented a role of viruses in the development and progression of periodontitis (Cappuyns et al., 2005). In particular, herpesviruses, a group of deoxyribonucleic acid (DNA) viruses, have emerged as putative periodontal pathogens (Slots, 2005). DNA from herpesviruses such as herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) has been detected in subgingival specimens (Contreras and Slots, 1996; Saygun et al., 2002; Saygun et al., 2004), gingival crevicular fluid (GCF)(Contreras and Slots, 1996; Parra and Slots, 1996), gingival tissue (Contreras et al., 2000; Ehrlich et al., 1983), and infiltrated immune cells from periodontitis sites (Contreras et al., 1999). In contrast, these herpesviruses could be found at the healthy sites but generally at a lower frequency as compared to periodontitis sites (Contreras et al., 2000; Contreras et al., 1999; Parra and Slots, 1996).

Innate immunity serves as the first line of defense against invading microorganisms. Innate antibacterial immune response in periodontal disease has been the focus of considerable recent research (Schenkein, 2006; Teng, 2006; Zasloff, 2002). However, to date there is relatively little information regarding periodontal innate antiviral immunity. Gingival epithelium, the outermost physical barrier of periodontium, plays important role as the local innate non-immune cells. It bears variety of sensing receptors called Toll-like receptors (TLRs) which recognize and activate inflammatory responses

not only to bacteria but also to viruses (Mahanonda and Pichyangkul, 2007). In addition to the cellular components, there are the non-cellular components such as secretory innate mediators with antibacterial properties that help protected periodontal mucosa. These include human α-defensins (commonly known as human neutrophil peptides, HNPs)(Goebel et al., 2000; Pisano et al., 2005; Puklo et al., 2008), human β-defensins (HBDs)(Diamond et al., 2001; Mathews et al., 1999), cathelicidin (LL-37)(Murakami et al., 2002; Puklo et al., 2008), lactoferrin (H. M. Friedman, 2006; McNeely et al., 1995), and secretory leukocyte protease inhibitor (SLPI)(Into et al., 2006; McNeely et al., 1995) which could be detected in GCF and saliva. Interestingly, these anti-bacterial proteins also have antiviral properties.

It is known that SLPI, protein kinase R (PKR), 2',5'-oligoadenylate synthetase (OAS), and Myxovirus resistance A (MxA) are mucosal proteins which are implicated in antiviral activity (Franken et al., 1989; Milush et al., 2007; Santoro et al., 2005; Vijay-Kumar et al., 2005). PKR, OAS, and MxA are induced by type I interferon (IFN) (Frese et al., 1996; Samuel, 2001). So far there has been no report of these innate antiviral proteins, except for SLPI, in periodontal tissue. In this study, we investigated messenger ribonucleic acid (mRNA) expression of different antiviral proteins (MxA, PKR, OAS, and SLPI) in periodontitis and compared to healthy tissue. Due to the availability of MxA monoclonal antibody in our laboratory, further investigation of MxA protein in periodontal biopsies by immunostaining was carried out.

1.2 Objectives

We explored the innate antiviral immunity in periodontal tissue. Our specific aims were:

1.2.1 To investigate mRNA expressions of different antiviral protein such as MxA, PKR, OAS, and SLPI in periodontitis tissues and to compare to those expressions

in healthy tissue by real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR).

1.2.2 To investigate the MxA expressions at the protein level by immunostaining in periodontitis tissues and to compare to those expressions in healthy tissue.

1.2.3 If MxA protein could be detected in periodontal tissue, the presence of type I IFN would be examined by QRT-PCR.

1.3 Hypothesis

1.3.1 mRNA of MxA, PKR, OAS, and SLPI antiviral protein is expressed in periodontal tissue samples. These antiviral protein expression in periodontitis are different from those in healthy.

1.3.2 Immunostaining of MxA protein is positive in periodontal tissue. MxA expression in periodontitis is different from those in healthy.

1.3.3 mRNA of Type I IFN is expressed in the periodontal tissue sample.

1.4 Field of research

Exploratory study of the antiviral protein expression including MxA, PKR, OAS, and SLPI in periodontal tissue.

1.5 Criteria inclusions

1.5.1 Periodontal tissue biopsies were obtained from healthy adult subjects.

1.5.2 Subjects who had clinically healthy periodontium with probing depth less than 4 mm were included.

1.5.3 Subjects who had severe periodontitis with probing depth 6 mm or more, severe bone loss and hopeless periodontal prognosis were included.

1.5.4 Analysis of antiviral protein expressions (MxA, PKR, OAS, and SLPI) was determined by QRT-PCR.

1.5.5 Pattern of MxA expression in gingival biopsy was determined by immunohistochemical staining.

1.5.6 Analysis of type I IFN was determined by QRT-PCR.

1.6 Limitation of research

This study cannot investigate many periodontal tissue samples due to limited numbers of periodontal biopsies and high expenses.

1.7 Application and expectation of research

1.7.1 New scientific information of different antiviral protein expression in disease and healthy periodontal tissue to provide novel insight into the role of innate antiviral protein in periodontal disease.

1.7.2 Publication in the international peered-reviewed journal.

1.8 Keywords

Antiviral protein, Innate immunity, Periodontal tissue, MxA

CHAPTER II

LITERATURE REVIEW

2.1 Viruses in periodontal disease

Periodontal inflammation represents the host response to bacterial plaque. The severity of periodontal diseases is somewhat dependent on a dynamic equilibrium of bacteria–host interactions (Darveau et al., 1997; Page et al., 1997). As periodontitis progresses, the supporting tissues of the teeth, including the periodontal ligament and alveolar bone, are destroyed, ultimately leading to tooth loss in severe cases. Although it is well recognized that the primary etiological factor in periodontal disease is the presence of gram-negative bacteria, the host immune response to these bacteria is of fundamental importance. Also, a number of local and environmental factors such as smoking, recent viral infections and physical and mental stress are thought to influence disease expression (Seymour, 1991).

In addition to bacteria, several studies have reported the role of virus in the development and progression of periodontitis. In particular, herpesviruses have emerged as putative periodontal pathogens (Slots, 2005). DNA from herpesviruses such as HSV-1,2, HCMV, and EBV has been detected in subgingival plaque, gingival tissue, infiltrated immune cells and GCF from periodontitis sites (Contreras et al., 2000; Contreras and Slots, 1996; Contreras et al., 1999; Ehrlich et al., 1983; Parra and Slots, 1996; Saygun et al., 2002). Additionally, HBV, HCMV and EBV have been frequently reported for their involvement in some forms of periodontal diseases such as acute necrotizing ulcerative gingivitis (ANUG)(Contreras et al., 1997) ,chronic periodontitis (Saygun et al., 2002), advanced periodontitis (Parra and Slots, 1996), aggressive periodontitis(Saygun et al., 2004; Yapar et al., 2003), apical periodontitis lesions

(Saboia-Dantas et al., 2007), and periodontal abscess (Saygun et al., 2004). In addition to herpesvirus group, human papilloma virus (HPV)(Madinier et al., 1992; Parra and Slots, 1996) and human immunodeficiency virus (HIV)(Contreras and Slots, 1996; Parra and Slots, 1996) have also been identified at periodontal disease sites. Also, an association has been demonstrated between HIV infection and necrotizing periodontal lesions and there are some reports of increased prevalence and severity of chronic periodontitis in HIV-positive subjects suggests that HIV infection predispose to chronic periodontitis (Cappuyns et al., 2005). Additionally, DNA from HSV, EBV and HCMV could also be found at the healthy sites but generally at a lower frequency as compared to periodontitis sites (Contreras et al., 2000; Contreras et al., 1999; Parra and Slots, 1996). Little is known about immune response to viruses in oral cavity.

2.2 Innate immunity in periodontal tissue

Innate immunity serves as the first line of defense against invading microorganisms. In oral cavity, saliva plays a key role in reducing accessibility of microbe-susceptible cells. It lubricates the oral cavity, also contains a variety of molecules with antimicrobial properties such as mucins (Oppenheim et al., 2007) amylase (Oppenheim et al., 2007), proline-rich proteins (Oppenheim et al., 2007), histatins (White et al., 2009), statherin (White et al., 2009), salivary gp-340 (or salivary agglutinin)(Oppenheim et al., 2007), lysozyme (McNeely et al., 1995), HNPs (Goebel et al., 2000), HBDs (Mathews et al., 1999), LL-37 (Murakami et al., 2002), thrombospondins (Crombie et al., 1998), lactoferrin (McNeely et al., 1995), and SLPI (McNeely et al., 1995). Most studies have focused on the antibacterial activities of these salivary soluble substances. However, many of them have been reported to have antiviral properties as well. For example, HBDs inhibit HSV-2 and HIV-1 (Hazrati et al., 2006; Quinones-Mateu et al., 2003), HNPs (Buck et al., 2006; Chang et al., 2003; Chang et al., 2005; Daher et al., 1986; Hazrati et al., 2006; John et al., 2005; Mackewicz et al., 2003; White et al., 2009; L. Zhang et al., 2002) and lactoferrin (Andersen et al., 2001;

Drobni et al., 2004; Harmsen et al., 1995; Hasegawa et al., 1994; Marchetti et al., 2009; Marchetti et al., 2004; Puddu et al., 1998; Valimaa et al., 2009) inhibit HSV-1,2 , HIV-1, HCMV, influenza A virus and papillomavirus , LL-37 inhibits HIV-1 and papillomavirus (Bergman et al., 2007; Buck et al., 2006) , and SLPI inhibits HSV-2, HIV-1, and influenza A virus (Beppu et al., 1997; John et al., 2005; Py et al., 2009; Skott et al., 2002; Wahl et al., 1997).

In periodontal tissue, gingival epithelium is constantly exposed to multiple assaults by microbes that live harmoniously in the oral niche, but most individuals maintain healthy homeostasis suggesting so effective innate immune response. It is a specialized, stratified squamous epithelium that is delineated and compartmentalized as oral epithelium, sulcular epithelium, and junctional epithelium (Lu et al., 2004). Gingival epithelium has a critical role in innate immune response by not only its physical barrier function, but also recognizing pathogen by TLRs and its antimicrobial properties that biologically suppress the propagation of putative pathogens (Diamond et al., 2001; Mahanonda et al., 2009). Moreover, junctional epithelium forming the attachment of soft tissue to the surface of the tooth (Schroeder and Listgarten, 1997) offer a pathway for migration of neutrophils into the gingival crevice in response to infection (Darveau et al., 1997; Schroeder and Listgarten, 1997).

The sulcus contains GCF in minute amounts that is a complex mixture of substances derived from serum, leukocytes, structural cells of the periodontium, and plaque bacteria (Uitto, 2003). Detailed analysis show the presence of serum albumin (Pisano et al., 2005), lactoferrin (S. A. Friedman et al., 1983), lysozyme (S. A. Friedman et al., 1983), HNPs (Pisano et al., 2005; Puklo et al., 2008), HBDs (Diamond et al., 2001), LL-37 (Puklo et al., 2008), SLPI (Into et al., 2006), cystatin A (Pisano et al., 2005), statherin (Pisano et al., 2005), and other unidentified components (Pisano et al., 2005). In periodontal health, the presence of HNPs, HBDs, LL-37, SLPI, lactoferrin, and cystatin in GCF suggests an ongoing innate antibacterial and antiviral process.

2.3 Antiviral activity in periodontal tissue

Little is known about the expression of antiviral protein in oral cavity. A variety of non-cellular innate mediators including HNPs (Goebel et al., 2000), HBDs (Mathews et al., 1999), LL-37 (Murakami et al., 2002), lactoferrin (McNeely et al., 1995), and SLPI (McNeely et al., 1995) are the examples of innate antiviral immunity present in saliva and GCF. SLPI, a member of the trappin gene family that includes elafin (Schalkwijk et al., 1999), is a 107-kiloDalton (kDa) protein produced and secreted primarily from epithelial cells lining mucosal surfaces (Franken et al., 1989) and skin (Sorensen et al., 2003), neutrophils (Sallenave et al., 1997), lipopolysaccharide-stimulated macrophages (Jin et al., 1997) and cultured human gingival keratinocytes (Jana et al., 2005; Westin et al., 2002). SLPI is a potent inhibitor of serine proteases (Thompson and Ohlsson, 1986), and participates in the mucosal defense including by reducing inflammation (Hiemstra, 2002); suppressing matrix metalloproteinase (MMP) production and activity (Y. Zhang et al., 1997); blocking the in vitro growth of selected bacteria (Hiemstra et al., 1996), fungi (Tomee et al., 1997), and non-HIV-1 viruses (Beppu et al., 1997); promoting fertility(Ota et al., 2002); and enhancing wound healing in skin (Ashcroft et al., 2000; Zhu et al., 2002). Furthermore, SLPI in GCF has been shown to promote the healing of periodontal tissue after non-surgical treatment in chronic periodontitis patients (Nakamura-Minami et al., 2003). Recent reports document constitutive expression of SLPI in oral epithelial cells of both healthy (Jana et al., 2005) and periodontitis tissues (Into et al., 2006). Levels of SLPI significantly increase after *in vitro* exposure of gingival epithelial cells to HIV (Jana et al., 2005).

At least three major proteins implicated in antiviral activity, such as PKR, OAS, and MxA, are induced by IFN-α and β (Frese et al., 1996; Samuel, 2001). Type I IFNs are important mediators of innate immune responses and critical for restricting early replication and spread of viruses (Le Bon and Tough, 2002). Humans with genetic defects in IFN signaling die of viral disease at an early age (Haller et al., 2007). IFNs are

produced by cells in direct response to virus infection. There is some evidence indicates that the expression of IFN-α1 mRNA was higher in periodontitis lesions compared with gingivitis lesions (Kajita et al., 2007). However, the overall expression level was low and no data available in healthy tissue.

When type I IFNs bind to and activate IFN receptor, the receptors signal to the nucleus through Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway and activate the expression of numerous IFN-stimulated genes (ISGs)(Haller et al., 2007) which have antiviral, antiproliferative, and immunomodulatory functions (de Veer et al., 2001; Der et al., 1998). Three IFN-induced enzyme systems representing major antiviral pathways such as PKR (Garcia et al., 2006; Williams, 1999), the 2',5'-OAS/RNaseL system (Silverman, 1994) and the Mx GTPases (Haller and Kochs, 2002) have been extensively studied. The double stranded ribonucleic acid (dsRNA)-activated protein kinase PKR is a ubiquitously expressed serine/threonine protein kinase that is induced by IFN (Stark et al., 1998) and activated by dsRNA, cytokine, growth factor and stress signals(Tan and Katze, 1999). Upon binding to dsRNA, this leads to the phosphorylation of its substrate, eukaryotic initiation factor 2 alpha (eIF2α), which inhibits the guanosine nucleotide exchange factor, eIF2β, and halts viral replication (Huang and Schneider, 1991; O'Malley et al., 1986). PKR may act by shutting down protein synthesis following infection of a cell and limit the transmission of virus to uninfected cells (Clemens, 1997).

2',5'-OAS, constitutively expressed in normal cells in a latent inactive form, is a marker of immune activation in hepatitis C (Gramenzi et al., 2005; McHutchison et al., 2007; Thimme et al., 2002). Upon binding to dsRNA, IFN-α induces the 2',5'-OAS which catalyses the formation of 2',5'-linked oligoadenylate and activates RNaseL, then breaks down viral and cellular RNA (Castelli et al., 1998; Pestka et al., 1987). PKR and OAS expression in oral cavity has not been reported.

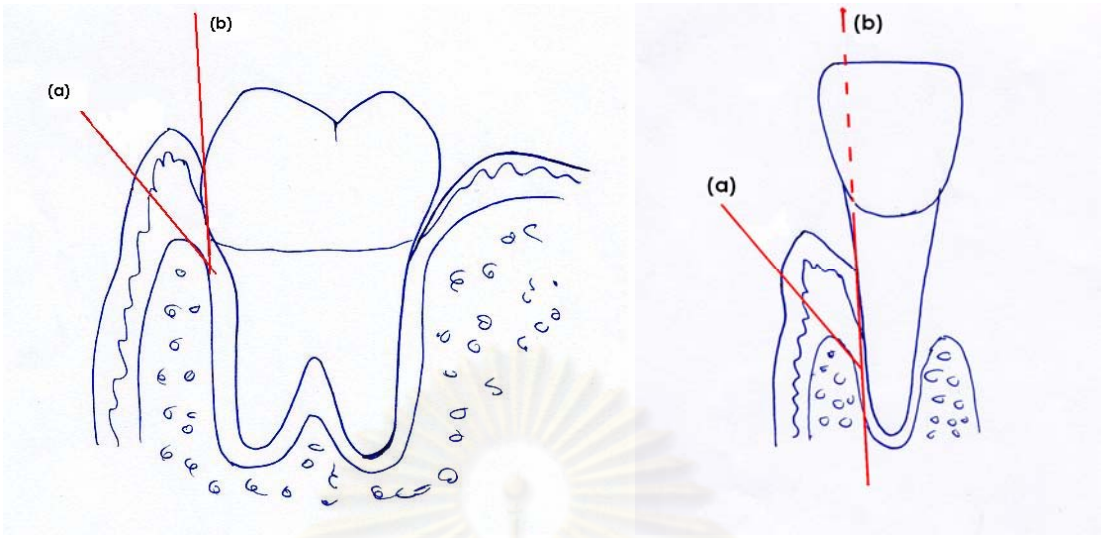
Among the known IFN-induced antiviral mechanisms, MxA proteins expression is strictly controlled in a dose-dependent manner by type I IFNs. They belong to the class of dynamin-like large guanosine triphosphatases (GTPases) and are known to be involved in intracellular vesicle trafficking and organelle homeostasis (Haller and Kochs, 2002; Kochs et al., 2002; Sever et al., 2000). The human MxA accumulates largely in the cytoplasm and remains in subcompartment of the endoplasmic reticulum (Accola et al., 2002; Stertz et al., 2006). Several properties of some Mx GTPases are self-assembly, association with intracellular membranes, and their antiviral activity against diverse viruses, including influenza virus, Thogoto virus, vesicular stomatitis virus (VSV), measles virus, bunyavirus, Semliki Forest virus, African swine fever virus (ASFV) and hepatitis B virus (HBV)(Frese et al., 1996; Frese et al., 1995; Gordien et al., 2001; Landis et al., 1998; Netherton et al., 2009; Pavlovic et al., 1990; Schwemmle et al., 1995; Yu et al., 2008; Zhao et al., 1996). Low basal levels of human MxA protein may be found in skin, certain cell lines, primary mononuclear cells, and Kupffer cells (Leifeld et al., 2001; Ronni et al., 1993; Shaker et al., 2009). There are strong expression of MxA proteins in Kupffer cells, lymphocytes, hepatocytes, cholangiocytes, and skin under pathological conditions (Fah et al., 1995; Leifeld et al., 2001; Shaker et al., 2009; Wenzel et al., 2005). Previous study demonstrated the expression of MxA in plasmacytoid dendritic cells infiltrated in oral mucosa lesions from oral lichen planus (Santoro et al., 2005). There have been no MxA studies so far in periodontitis. Since there is relatively little available information regarding periodontal innate antiviral immunity, we would like to be the first to detect the expression of different antiviral innate proteins, to include MxA, PKR, OAS, and SLPI in periodontal tissues, both in disease and health.

CHAPTER III

MATERIALS AND METHODS

3.1 Periodontal tissue samples

Periodontal tissue samples were collected from subjects who had clinically healthy periodontium and untreated severe chronic periodontitis lesion. The biopsies of healthy gingiva with probing depth less than 4 mm were obtained at the time of crown lengthening procedure for prosthetic reasons or impacted tooth removal. The biopsies of periodontitis lesion with gingival inflammation, evidence of bleeding on probing, radiographic evidence of bone loss, and probing depth not less than 6 mm were obtained at the time of removal the teeth that had hopeless periodontal prognosis and not affected by endodontic problem from Periodontal Clinic or Surgery Clinic, Faculty of Dentistry, Chulalongkorn University. The biopsies of both healthy and periodontitis tissue were prepared from surgical incision (Figure 1) according to appropriate treatment plan.



Healthy periodontium Periodontitis

Figure 1 Preparation of periodontal tissue biopsy from healthy and periodontitis tissue samples by (a) first internal bevel incision and (b) intrasulcular incision according to appropriate treatment plan.

The exclusion criteria for both groups included diabetes, bleeding disorders, gross oral pathology or treatment in the previous six months with antibiotics or anti-inflammatory drugs. Informed consent was obtained prior to inclusion in the study. The protocol was approved by the ethics committee of Faculty of Dentistry, Chulalongkorn University (No.21/2010). The excised tissues were immediately placed in either

3.1.1 Sterile tube containing RNAlater (500µl) (Qiagen, Chatsworth, CA, USA) for analysis mRNA expression by QRT-PCR or

3.1.2 Sterile dish containing phosphate-buffered saline (PBS)-soaked gauze for determination MxA protein by immunohistochemical staining.

3.2 mRNA expression of antiviral proteins and cytokine in periodontal tissues

To quantitate amount of MxA, PKR, OAS-1, SLPI, and IFN-α mRNA in the tissue, we used real-time reverse transcription polymerase chain reaction. Periodontal tissue samples kept in RNAlater were washed twice and total RNA were separated by using RNeasy Mini kit (Qiagen, USA) with proteinase K digestion and on-column DNAse treatment. 1 μg of total RNA was reverse transcribed using ImProm-II™ Reverse Transcription System for RT-PCR, according to the manufacturer's instructions (Promega, USA). Real-time PCR was performed on the LightCyclerTM (Roche Molecular Diagnostics) in a total volume of 20 μl containing 0.5 μM of each forward and reverse oligonucleotide primer pairs, 10 μl SYBR Green PCR Master Mix (FastStart Taq DNA polymerase, reaction buffer, deoxyribonucleoside triphosphate (dNTP) mix, SYBR Green I dye, and $MgCl_2$), 4 μl water, and 5 μl complementary DNA (cDNA) template. The primers were specific to a conserved region of MxA, PKR, OAS-1, SLPI, and IFN-α as shown in Table 1.

The temperature program for MxA, PKR, OAS-1, SLPI, and IFN-α consisted of an initial denaturation step at 95^0C for 10 min, followed by amplification of the template for 40 cycles of 95^0C for 10 sec, 60^0C for 10 sec, and 72^0C for 20 sec (single acquisition). After amplification was completed, a final melting curve was performed at 95^0C for 5 sec, 65^0C for 1 min, and heating to 97^0C using a ramp rate of 0.11^0C/sec with continuous monitoring of fluorescence. Determination of product specificity depended upon generation of specific PCR products with well-defined melting temperatures of: MxA 84^0C, PKR 79^0C, OAS-1 86^0C, SLPI 85^0C, and IFN-α 85^0C. Real-time fluorescence measurement was read and a threshold cycle (C_t) value for each sample was calculated by determining the point at which the fluorescence exceeds a threshold limit. Samples were defined as negative if the C_t values exceeded 35 cycles. The mRNA of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as internal control

in each sample to control sample to sample variations in RNA concentration. As a negative control, a PCR reaction was performed without template DNA. Peripheral blood mononuclear cell (PBMC) was used as a positive control. Expression of antiviral proteins mRNA levels was calculated by using the comparative Ct method ($2^{-[delta]Ct}$ formula) after normalization to GADPH.

Table 1: Primer sequences of antiviral proteins and cytokine and GAPDH

Product	Forward primer	Reverse primer	Amplicon size (bp)
MxA [$]	GCTACACACCGTGACGGATATGG	CGAGCTGGATTGGAAAGCCC	289
PKR [##]	GCCTTTTCATCCAAATGGAATTC	GAAATCTGTTCTGGGCTCATG	301
OAS-1 [**]	CATCCGCCTAGTCAAGCACTG	CCACCACCCAAGTTTCCTGTAG	309
SLPI [*]	TTCCCCTGTGAAAGCTTGATTC	GATATCAGTGGTGGAGCCAAGTC	106
IFN-α [&]	GGATGAGACCCTCCTAGACAAAT	ATGATTTCTGCTCTGACAACCTC	216
GAPDH [#]	GAAGGCTGGGGCTCATTT	CAGGAGGCATTGCTGATGAT	138

([$]primer sequence by Antonelli et al., 1999, [##] primer sequence by Farrugia and Cann, 1999, ** primer sequence by Kato et al., 2004, * primer sequence by Amigo et al., 2006, [&]primer sequence by Kato et al., 2003, [#]primer sequence by Carraro et al., 2005)

3.3 Immunohistochemical staining for MxA protein

Periodontal tissue samples kept in PBS-soaked gauze were rinsed once with PBS to remove blood clots and adherent erythrocytes before embedding in a gel like media known as Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Miles Inc., USA), snap frozen in liquid nitrogen and stored at -80°C until sectioning. After cutting, cryostat sections (4 µm) were transferred to SUPERFROST® PLUS microscope slides, air dried, and kept at -80°C until use.

For immunohistochemical staining using EnVision™ G/2 DoubleStain system (Dako,Denmark), tissue samples were shortly thawed at room temperature before fixing for 10 min in cold acetone. The samples were then air dried for 10 min, circled with PAP pen (Dako,Denmark) and rehydrated with PBS for 3 min. Thereafter, the sections were incubated in 0.3% hydrogen peroxide in PBS for 20 min in order to quench endogenous peroxidase activity. After washing with PBS, the sections were incubated with appropriate non-specific protein blocking buffer (5% skim milk) for 30 min at room temperature and then incubated with mouse immunoglobulin G (IgG) (Dako,Denmark) as negative control, primary antibody against human MxA (dose 1:100, Department of virology ,Institute for Medical Mikrobiology & Hygiene, Freiburg, Germany) or primary antibody against CD3 (dose 1:100, BD Biosciences, USA) in blocking buffer at 37°C for 60 min. After being washed 3 times with PBS, the sections were incubated with 100 µl Horseradish peroxidase (HRP) labeled polymer for 30 min, washed 3 more times with PBS and then reacted with diaminobenzidine (DAB) chromagen. Once optimal color intensity was obtained, reaction was halted by immersing the tissue sections in double distilled water. The sections were then counterstained with hematoxylin for 1 min, rinsed in distilled water twice and mounted using Dako Cytomation Paramount Aqueous Mounting Medium.

Digitalized images were obtained using a light microscope and camera system (BX50 and C-5060; Olympus, Japan). Semiquantitative analysis was performed on three selected high-power fields (X400) within oral epithelium including gingival crest area, middle part, and lower border of each section, in which the cell or nucleus outline could be seen in epithelial layer, if possible, as shown in Figure 2. Cells were judged positive by their brownish cytoplasmic staining. Calibration for counting MxA positive cells was carried out by two examiners before the study. The weighted kappa coefficient between examiners was 0.91. The value for repeated measurements within examiner was 0.90. Therefore, data collection was performed by a single calibrated investigator. The results of Kappa statistic test were presented in Appendix A and B. The immunoreactivity was scored with semiquantitative scoring method: score 1–3: the areas of positive cells were less than 10%, 10–50%, and more than 50% of oral epithelium, respectively. The median score of three selected fields was used as representative of individual subjects.

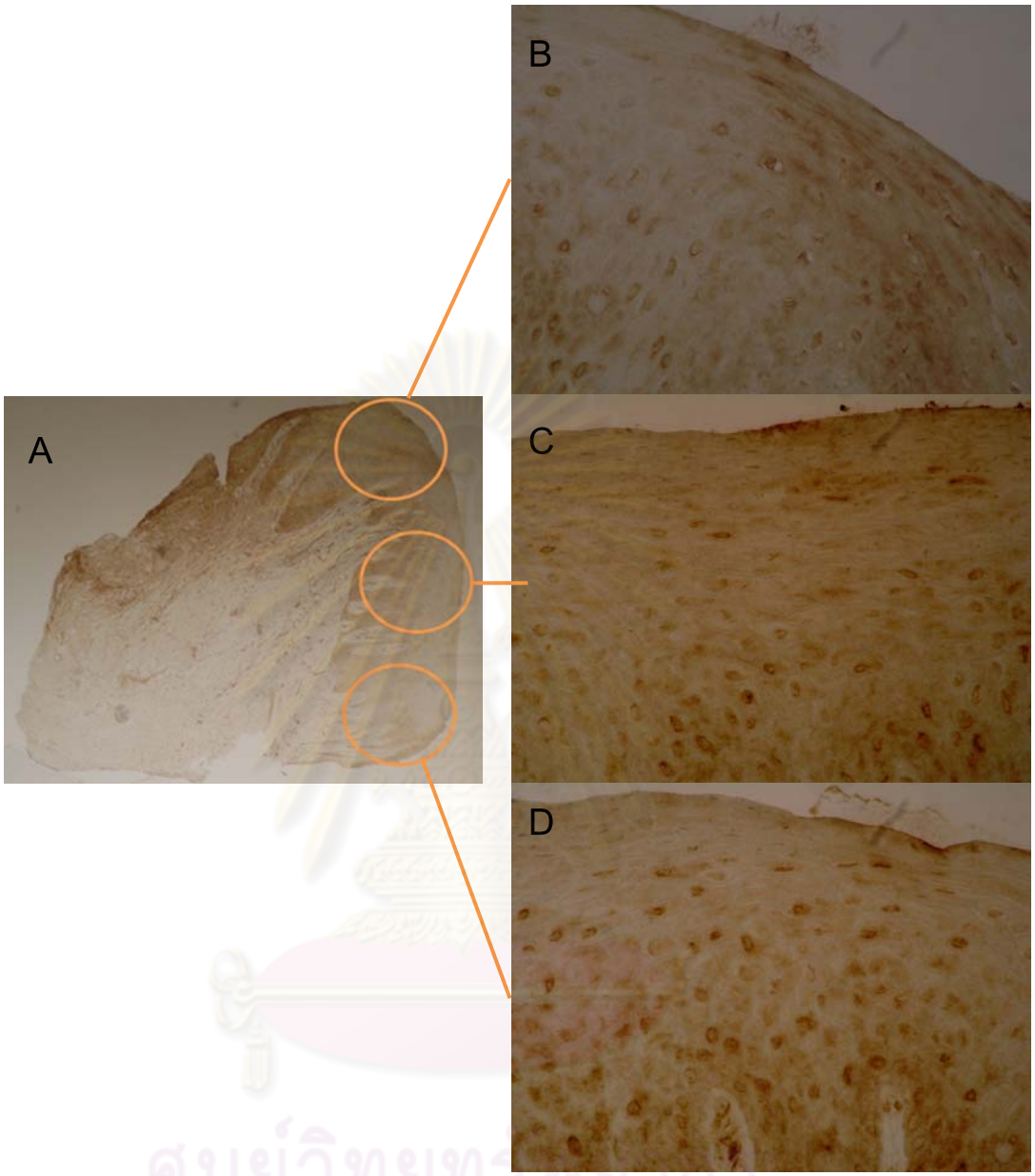


Figure 2 Sampling of three examined areas within oral epithelium from each section. (A) At magnified x40, three selected areas as shown as circles were obtained from gingival crest, middle part, and lower border of each section. (B, C, and D) were high power image (magnification x400) from gingival crest, middle part, and lower border, respectively.

3.4 Statistic analysis

Statistical comparisons between healthy and periodontitis group with respect to mRNA expression of antiviral proteins and cytokine as well as difference in the distribution of MxA protein-staining score were analyzed using SPSS V17.0 software. The nonparametric Mann-Whitney U-test was used. A *P*- value of < 0.05 was considered as statistically significant.

CHAPTER IV

RESULTS

4.1 mRNA Expression of antiviral proteins in periodontal tissue

Antiviral proteins are expressed on many cells and known as non-cellular compartment of the innate immune system. Here we investigated mRNA expression of different antiviral proteins, MxA, PKR, OAS-1 and SLPI in periodontal tissue specimens. The specimens were collected from five periodontitis patients (mean age: 51.2 $\pm$ 10.7 years; range 35-64) and five healthy patients (mean age: 24.8 $\pm$ 14.2 years; range 12-48). The mean probing depth of the periodontitis and healthy sites were 8.4 $\pm$ 1.5mm (range 7–10 mm) and 2.4 $\pm$ 0.5 mm (range 2-3 mm), respectively. Descriptive profile of gingival biopsy samples was presented in Appendix C. Total RNA from periodontal tissue was analyzed by QRT-PCR using specific primers. We found the mRNA expression of MxA, PKR, and OAS-1 in all examined specimens (Figure 3). Comparison of mRNA expression levels of MxA, PKR, and OAS-1 relative to GAPDH between healthy and periodontitis did not demonstrate any significant differences (P = 0.917 for MxA, P = 0.463 for PKR, and P = 0.465 for OAS-1). The median relative expression of MxA, PKR, and OAS-1 ranged from 0.91-1.23. High SLPI expression was observed in all samples, ranging from 23.92-99.04 and no differences in expression between disease statuses were detected (P = 0.754 for SLPI, Figure 3). Data of anti-viral protein mRNA expression in individual subjects (n=10) were presented in Appendix D, E, F and G. The results of Mann-Whitney U-test were presented in Appendix H.

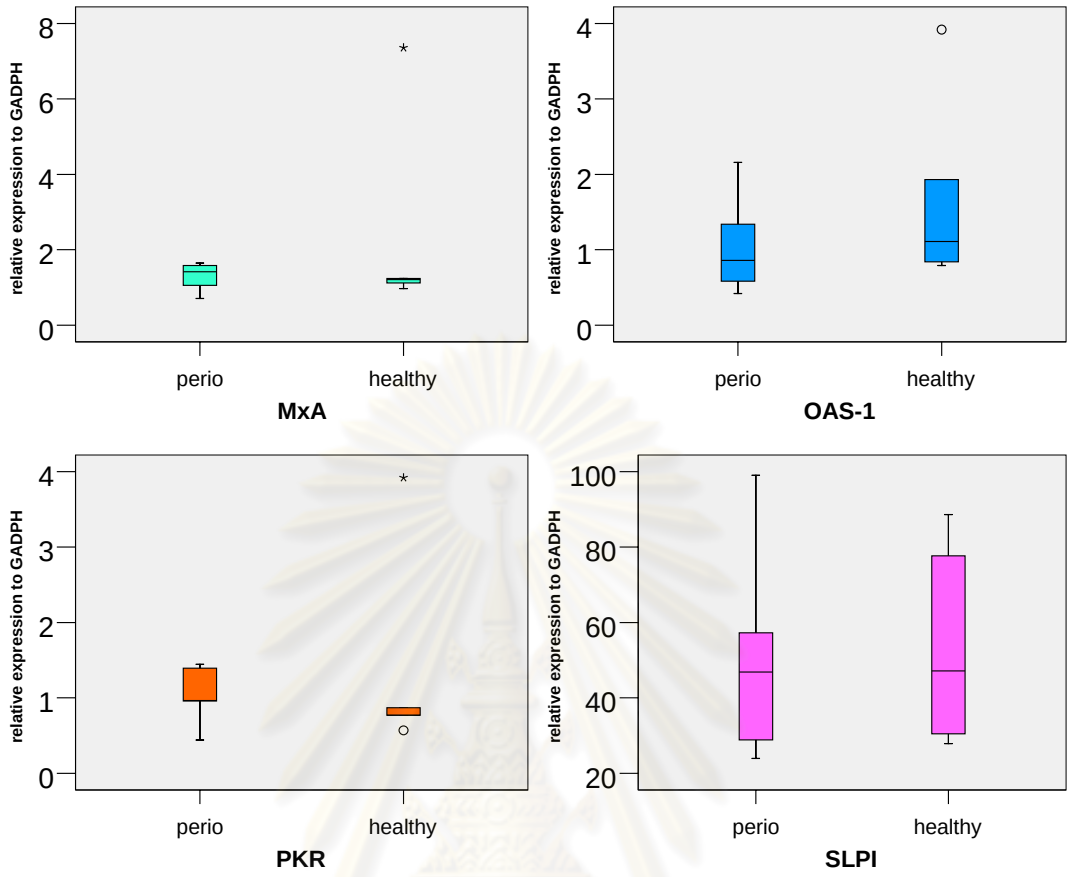


Figure 3 Comparison of the relative gene expressions of antiviral proteins between periodontitis and healthy tissues by QRT-PCR (n=5 in each group). The relative quantity of mRNA was normalized to the relative quantity of GAPDH. The box plots showed medians, 25th and 75th percentiles as boxes, and minimum and maximum values as whiskers. Outlying values are shown as open circles and extreme values as asterisk. No significant differences for all the antiviral proteins examined were observed between periodontitis and healthy.

4.2 Immunohistochemical analysis of MxA protein in periodontal tissue

Due to the mRNA expression of antiviral proteins in all periodontal tissue specimens and MxA protein has been well recognized to have antiviral activity against both RNA and DNA virus (Frese et al., 1996; Frese et al., 1995; Gordien et al., 2001; Landis et al., 1998; Netherton et al., 2009; Pavlovic et al., 1990; Schwemmle et al., 1995; Yu et al., 2008; Zhao et al., 1996), further investigation to confirm MxA expression at the protein level in periodontal biopsies by immunostaining was carried out. The specimens were collected from seven periodontitis patients (mean age: 52.1 $\pm$ 13.9 years; range 36-77) and nine healthy patients (mean age: 31.9 $\pm$ 14.1 years; range 20-67). The mean probing depth of the periodontitis and healthy sites were 8.3 $\pm$ 1.1mm (range 7–10 mm) and 2.1 $\pm$ 0.8 mm (range 1-3 mm), respectively. Descriptive profile of gingival biopsy samples was presented in Appendix C.

Immunohistochemical data in Figure 4 confirmed the results of mRNA expression of MxA in all healthy and periodontitis tissue specimens. MxA expression was especially strong in epithelial layer while some positive cells such as macrophage-like cells were identified in connective tissue. Epithelial MxA immunoreactivity was stronger in basal, spinous, and granular than outermost layer of oral epithelium. Most of periodontitis tissue samples consisted of MxA positive cells 10-50% or >50% in the oral epithelium whereas a majority of epithelial cells in healthy samples stained positively for MxA. For comparison between periodontitis and healthy tissues, semiquantitative scores were described for each MxA staining tissues which were presented in Table 2. There was significantly higher score of MxA immunostaining in the healthy group than periodontitis group (P= 0.012), thus highlighting the role of MxA in healthy periodontal tissue. Data of immunostaining scores of MxA in individual subjects (n=16) were presented in Appendix I. The results of Mann-Whitney U-test were presented in Appendix J.

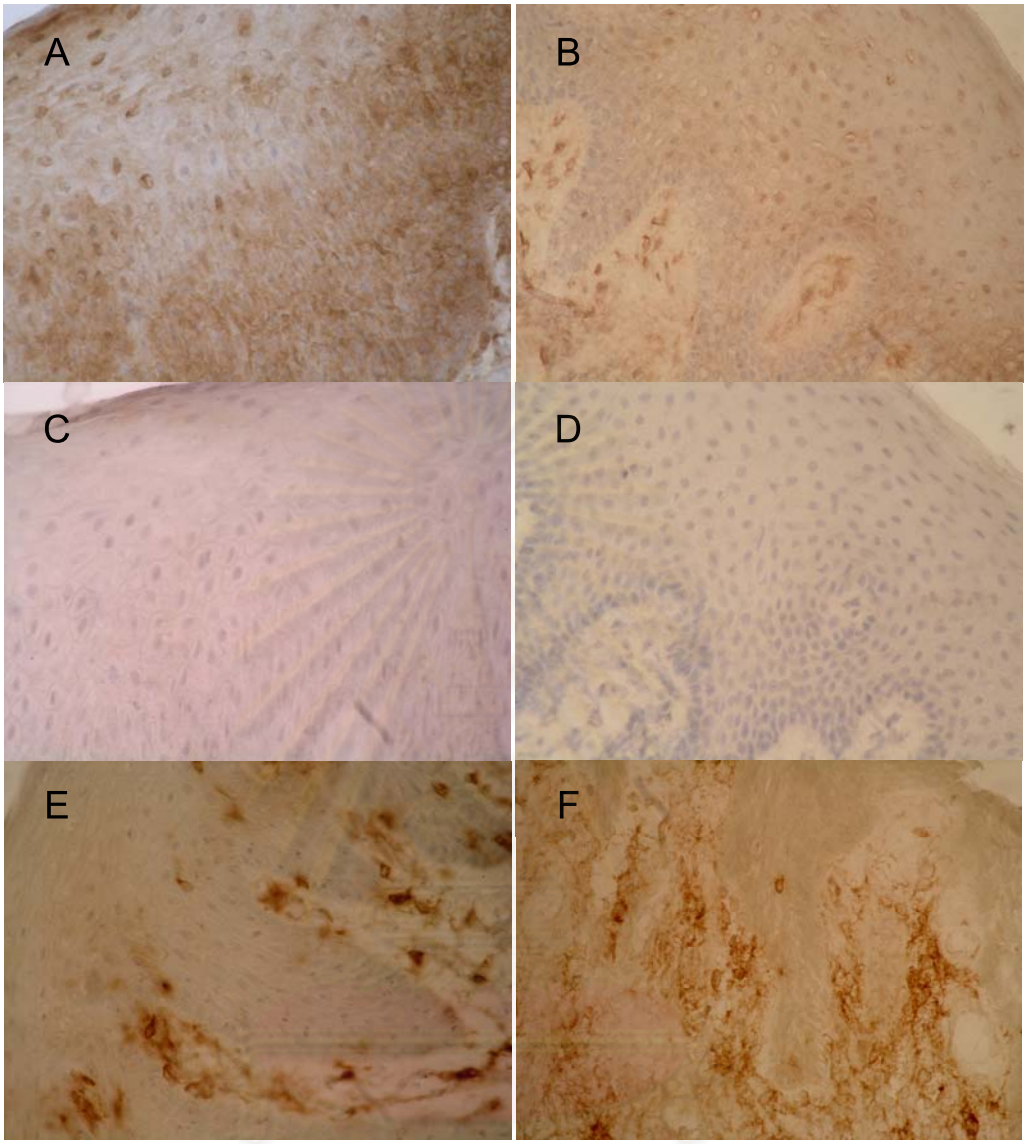


Figure 4 Representative immunohistochemical finding of MxA protein expression in periodontal tissue. (A) The oral epithelium of healthy tissue. (B) The oral epithelium of periodontitis tissue. MxA expression was stained with DAB and is shown in brown. Cell nuclei were counterstained with hematoxylin and are shown in blue-purple. MxA staining was positive in both healthy and periodontitis tissue. Negative control section of healthy (C) and periodontitis tissue (D) were stained with non-specific IgG. Positive control section of healthy (E) and periodontitis tissue (F) were stained with primary antibody against CD3. (Magnification x400)

Table 2 : The immunoreactive staining scores of MxA protein in oral epithelium of periodontal tissue specimens.

Group	Immunostaining score			
	0 n (%)	1 n (%)	2 n (%)	3 n (%)
Periodontitis	0	1 (14)	3 (43)	3 (43)
Healthy	0	0	0	9 (100)

Note: score 1 = the area of positive cells in oral epithelium was less than 10%, score 2 = 10–50%, and score 3 = more than 50%.

In healthy periodontal tissue, the gingival sulcus is a shallow, v-shaped crevice. It is bounded by the tooth surface on one side and the sulcular epithelium on the other. At the bottom lies the junctional epithelium which forms firmly attachment to the tooth/root surfaces. Our preliminary immunostaining results of epithelial MxA were from the oral side of periodontal tissue, but not from the sulcus side since epithelium at the sulcus side, especially junctional epithelium, always get torn during surgical procedure. In this study, we were able to obtain one healthy periodontal tissue specimen completed with oral, sulcular and junctional epithelium. We found that immunoreactivity of epithelial MxA protein was strong not only on the oral side of healthy periodontal tissue but also in the gingival sulcus area. It should be noted that junctional epithelium demonstrated very intense MxA expression, thus suggesting innate antiviral activity in healthy gingival sulcus. Also some MxA immunoreactivity was found in connective tissue layer, but to a much lesser extent (Figure 5).

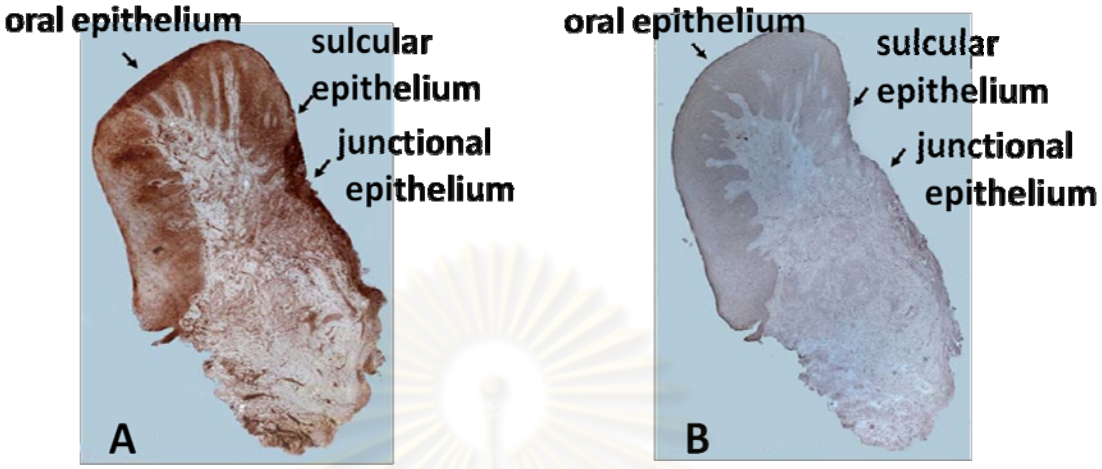


Figure 5 MxA protein expression in junctional, sulcular, and oral epithelium in a healthy periodontal tissue specimen (A). Negative control was stained with non-specific IgG (B).

4.3 mRNA Expression of IFN-α in periodontal tissue

Since MxA is known to be induced by type I IFN (Haller et al., 2007), we then investigated the presence of IFN-α in healthy periodontal tissue by QRT-PCR. We found negligible IFN-α expression (Figure 6). Data of cytokine, IFN-α, in individual subjects (n=5) were presented in Appendix D, E, F and G.

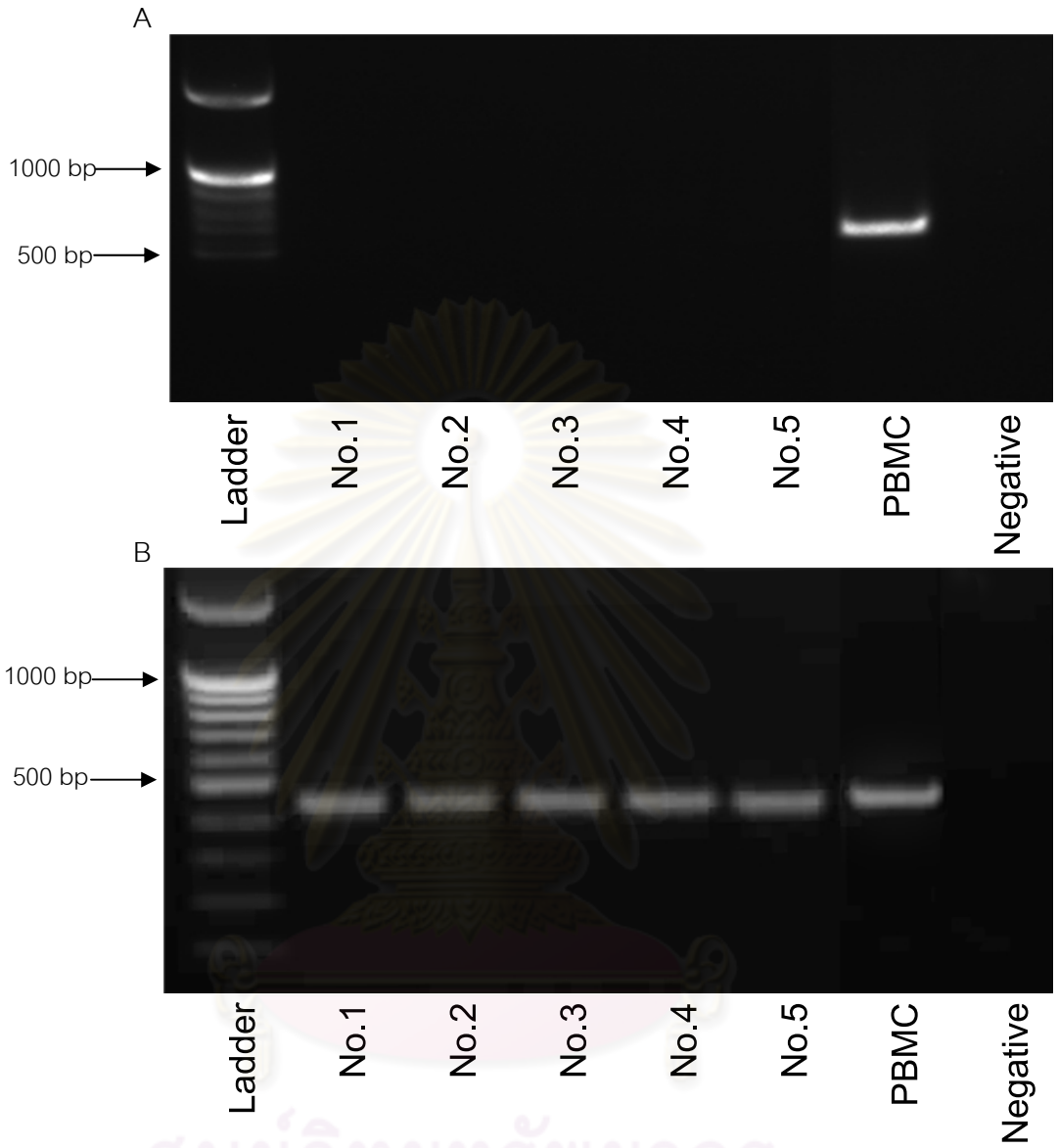


Figure 6 IFN-α expression in healthy periodontal tissues by RT-PCR (n=5) (A). GAPDH mRNA was used as an internal control (B). PBMC mRNA was used as positive control. Water was used as a negative control.

CHAPTER V

DISCUSSION AND CONCLUSION

Periodontal tissue is important in tooth support, mastication, and speech. Besides these familiar functions, recent data suggest a critical role of periodontal tissue in protective innate immune response against microbial infection (Mahanonda and Pichyangkul, 2007). Unlike considerable research in the area of innate antibacterial immune response, to date there is relatively little available information regarding innate antiviral immunity in periodontal tissue. In this study, we first demonstrated that periodontal tissue from both healthy and periodontitis expressed variety of antiviral proteins, MxA, PKR, OAS, and SLPI, thus suggesting the role of periodontal tissue in the antiviral innate immunity. Our finding of SLPI in periodontal specimens agreed with previous studies (Into et al., 2006; Jana et al., 2005). Expression levels of MxA, PKR, OAS, and SLPI were similar between healthy and periodontitis group.

MxA protein has been well recognized to have antiviral activity against both RNA and DNA virus (Frese et al., 1996; Frese et al., 1995; Gordien et al., 2001; Landis et al., 1998; Netherton et al., 2009; Pavlovic et al., 1990; Schwemmle et al., 1995; Yu et al., 2008; Zhao et al., 1996). Our immunohistochemical data demonstrated MxA proteins in all periodontal tissue specimens which supported our preliminary findings of MxA mRNA expression. MxA protein was remarkably strong in epithelial layer of both healthy and periodontitis. Epithelial MxA immunoreactivity was stronger in basal, spinous, and granular than outermost layer of oral epithelium. These basal, spinous, and granular layers consist of active, differentiating, and proliferating cells (Bartold et al., 2000). While no differences were found in MxA mRNA expression in the whole specimens (including epithelial and connective layers) of healthy and periodontitis tissues, we found significant differences in MxA protein levels in epithelial layers

between these two groups. By using semiquantitative scoring, the healthy group had a significant higher MxA score than periodontitis group (*P*= 0.012), thus highlighting the role of MxA protein in healthy periodontal tissue. Our result is the first report of MxA expression in healthy tissue in the oral cavity. Santoro et al. (2005) reported MxA expression in the oral mucosal lesion of lichen planus but they did not investigate the expression in healthy tissue. However, functional assay for the epithelial MxA protein is needed to confirm their defensive role in periodontal tissue.

MxA expression in healthy periodontal tissue is very interesting since it is well known that this protein is induced by type I IFN (Haller et al., 2007). From our observation, there was negligible expression of IFN-α in healthy periodontal tissue. What induces MxA expression in periodontal tissues, particularly at the epithelial layer, requires further research. It may be other local mediators that constitutively expressed in healthy periodontal tissues that are capable of inducing MxA protein. For example, antimicrobial peptides including HNP, HBD, and LL37 which have been detected in healthy periodontal tissues and GCF, may be good candidates for MxA inducer (Diamond et al., 2001; Pisano et al., 2005; Puklo et al., 2008).

One healthy periodontal specimen which was consisted of gingival sulcus area demonstrated strong MxA protein not only in the oral epithelium but also in the sulcular and junctional epithelium. It should be noted that the junctional epithelium showed very intense MxA protein. Research in recent years has shown that the junctional epithelium may play a much more active role in innate immunity than previously thought. Junctional epithelial cells expressed adhesion molecule, intercellular adhesion molecule 1 (ICAM-1) and a potent chemokine for polymorphonuclear cell (PMN), interleukin-8 (IL-8) (Crawford and Hopp, 1990; Tonetti et al., 1998). The constant recruitment and migration of PMN from subepithelial connective tissue blood vessels through the junctional epithelium into the sulcus is a phenomenon in healthy gingival sulcus (Schroeder, 1973). The area of intense MxA staining in the sulcus is the same pathway of PMN migration,

hence PMN products may be released and induce MxA expression in junctional epithelium. This speculation requires further investigation.

In conclusion, our study demonstrated variety of antiviral proteins expression: MxA, PKR, OAS and SLPI in periodontal tissues, both healthy and periodontitis. Strong MxA protein expression in epithelial layer of healthy tissue has been observed, particularly at the strategic area: gingival sulcus. Our results suggest that periodontal tissue is equipped with different antiviral proteins which may play role in innate immunity against viruses, thus in part maintaining periodontal homeostasis. Further research is required to understand how these proteins are induced and function in periodontal tissues, both in health and disease.

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APPENDICES

Appendix A : Kappa statistic result of interexaminer reliabilities in scoring the immunoreactive MxA staining

Immunostaining score of	
Examiner 1	Examiner 2
3	3
3	3
3	3
3	3
3	3
3	3
3	3
3	3
2	2
1	1
3	2
2	2
2	2
1	1
2	2

Crosstabs

Row variable (first classifier) : A

Column variable (second classifier): B

	1	2	3
1	2	0	0
2	0	4	0
3	0	1	8

General agreement over all categories (2 raters)

Cohen's kappa (weighted by 1-abs(i-j)/(1-k))

ratings weighted by:

1	0.5	0
0.5	1	0.5
0	0.5	1

Observed agreement = 96.67%

Expected agreement = 63.78%

Kappa = .907975 (se = .207165)

95% confidence interval for kappa = .50194 to 1.314011

z (for kw = 0) = 4.382869

P < 0.0001

Disagreement over any category and asymmetry of disagreement (2 raters)

Marginal homogeneity (Maxwell) chi-square = * df = * *

Symmetry (generalised McNemar) chi-square = 1 df = 3 P = 0.8013

Appendix B : Kappa statistic result of intraexaminer reliabilities in scoring the immunoreactive MxA staining

Immunostaining score of	
Examiner 1A	Examiner 1B
3	3
3	3
3	3
3	3
3	3
3	3
3	3
3	3
2	3
1	1
3	3
2	2
2	2
1	1
2	2

Crosstabs

Row variable (first classifier) : A

Column variable (second classifier): B

	1	2	3
1	2	0	0
2	0	3	1
3	0	0	9

General agreement over all categories (2 raters)

Cohen's kappa (weighted by 1-abs(i-j)/(1-k))

ratings weighted by:

1	0.5	0
0.5	1	0.5
0	0.5	1

Observed agreement = 96.67%

Expected agreement = 65.11%

Kappa = .904459 (se = .210506)

95% confidence interval for kappa = .491874 to 1.317043

z (for kw = 0) = 4.296591

P < 0.0001

Disagreement over any category and asymmetry of disagreement (2 raters)

Marginal homogeneity (Maxwell) chi-square = * df = * *

Symmetry (generalised McNemar) chi-square = 1 df = 3 P = 0.8013

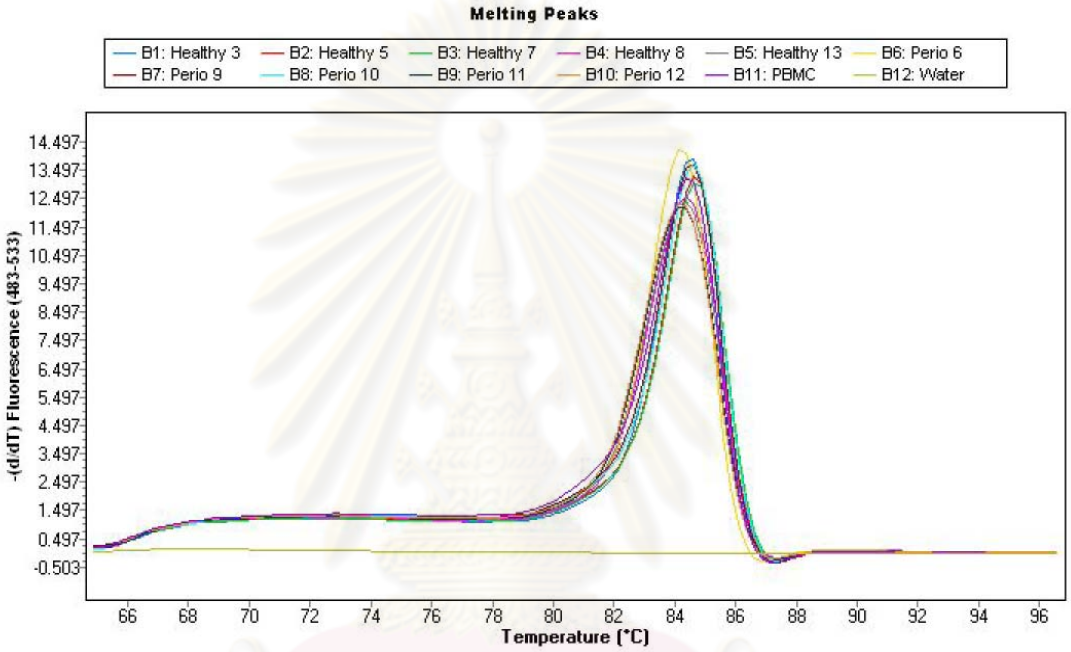
Appendix C : Descriptive profile of gingival biopsy samples

No.	Sex	Age (years)	Tooth No.	Probing depth (mm)	Group	Method
1	Female	20	#38	3	Healthy	QRT-PCR
2	Female	28	#21	2	Healthy	QRT-PCR
3	Male	48	#26	2	Healthy	QRT-PCR
4	Male	12	#14	2	Healthy	QRT-PCR
5	Female	16	#48	3	Healthy	QRT-PCR
6	Male	54	#15	8	Periodontitis	QRT-PCR
7	Male	55	#37	7	Periodontitis	QRT-PCR
8	Female	48	#27	10	Periodontitis	QRT-PCR
9	Female	64	#21	7	Periodontitis	QRT-PCR
10	Male	35	#41	10	Periodontitis	QRT-PCR
11	Female	67	#23	1	Healthy	IMMUNO
12	Male	20	#38	3	Healthy	IMMUNO
13	Male	26	#12	3	Healthy	IMMUNO
14	Female	33	#21	2	Healthy	IMMUNO
15	Female	27	#21	2	Healthy	IMMUNO
16	Female	34	#13	2	Healthy	IMMUNO
17	Female	20	#23	3	Healthy	IMMUNO
18	Male	30	#15	1	Healthy	IMMUNO
19	Female	30	#21	2	Healthy	IMMUNO
20	Male	60	#16	7	Periodontitis	IMMUNO
21	Male	77	#37	8	Periodontitis	IMMUNO
22	Male	36	#46	7	Periodontitis	IMMUNO
23	Male	51	#14	10	Periodontitis	IMMUNO
24	Male	49	#27	9	Periodontitis	IMMUNO
25	Female	54	#28	9	Periodontitis	IMMUNO
26	Female	38	#31	8	Periodontitis	IMMUNO

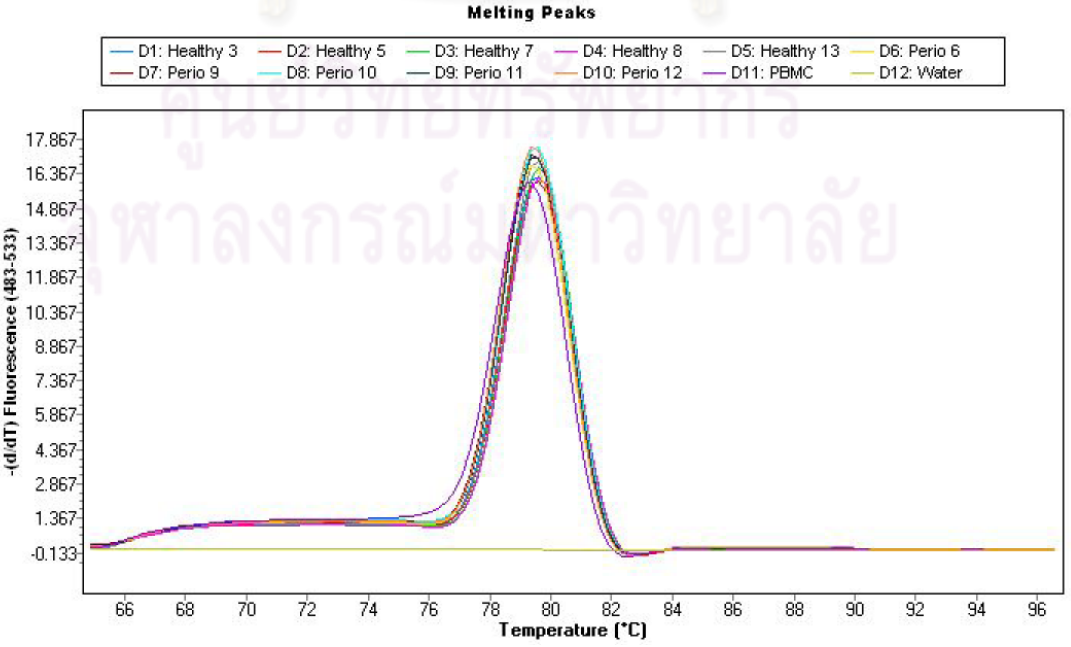
Appendix D : Melting curve analysis of amplified samples with cDNA derived from (D1) MxA, (D2) PKR, (D3) OAS-1, (D4) SLPI , and (D5) IFN-α.

As a negative control, template DNA was replaced by PCR- grade water.

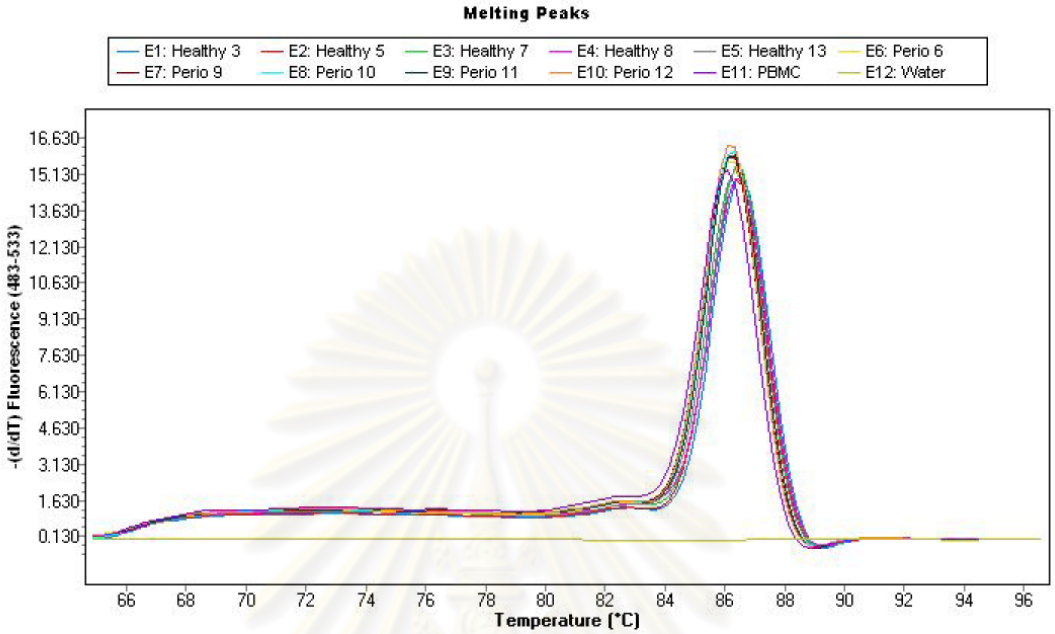
D1



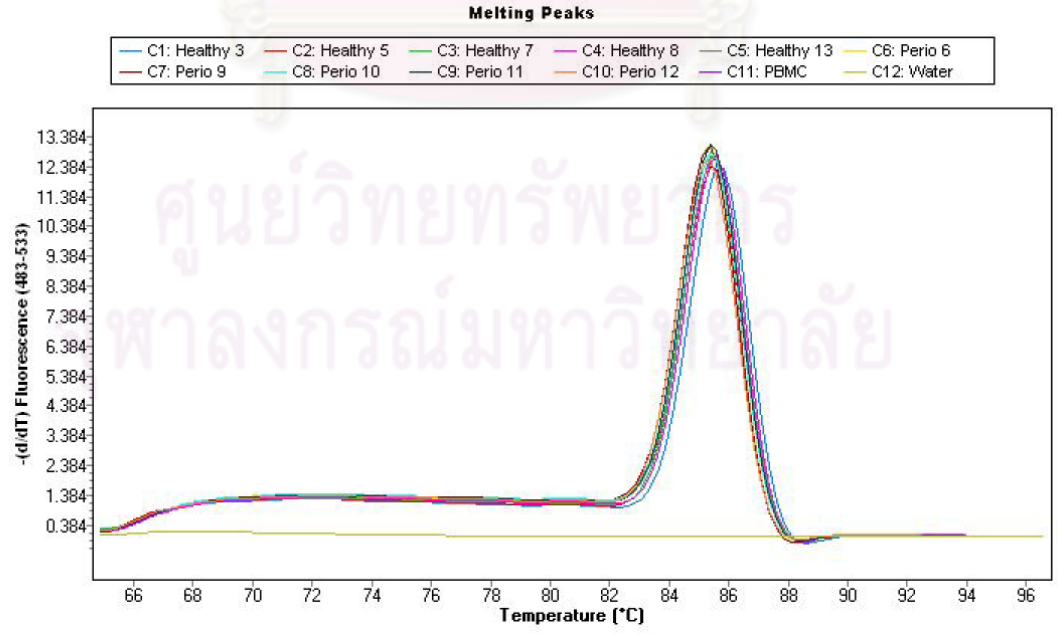
D2



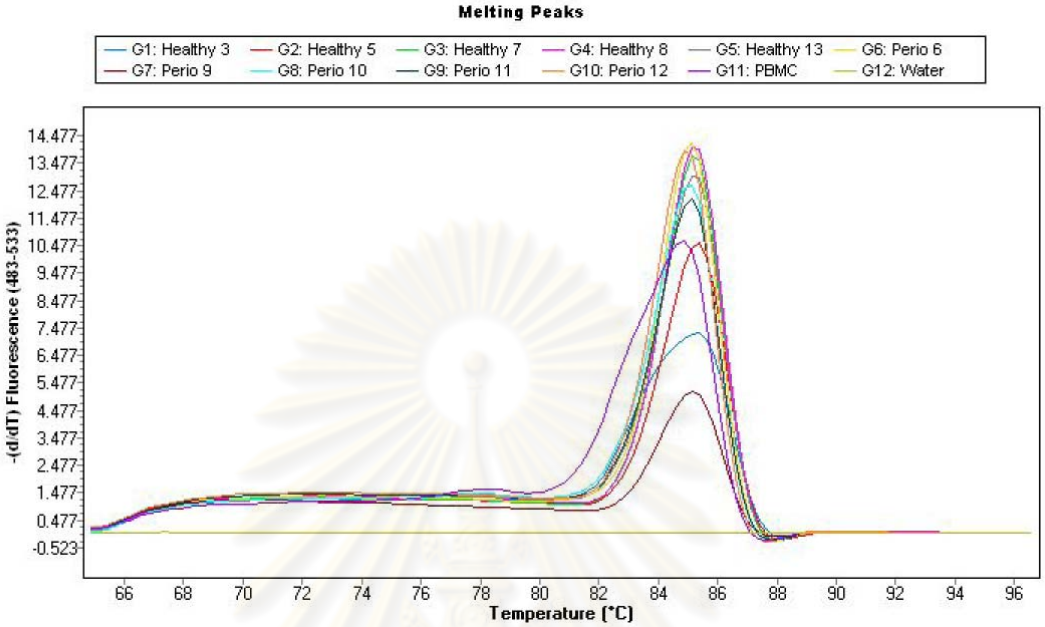
D3



D4



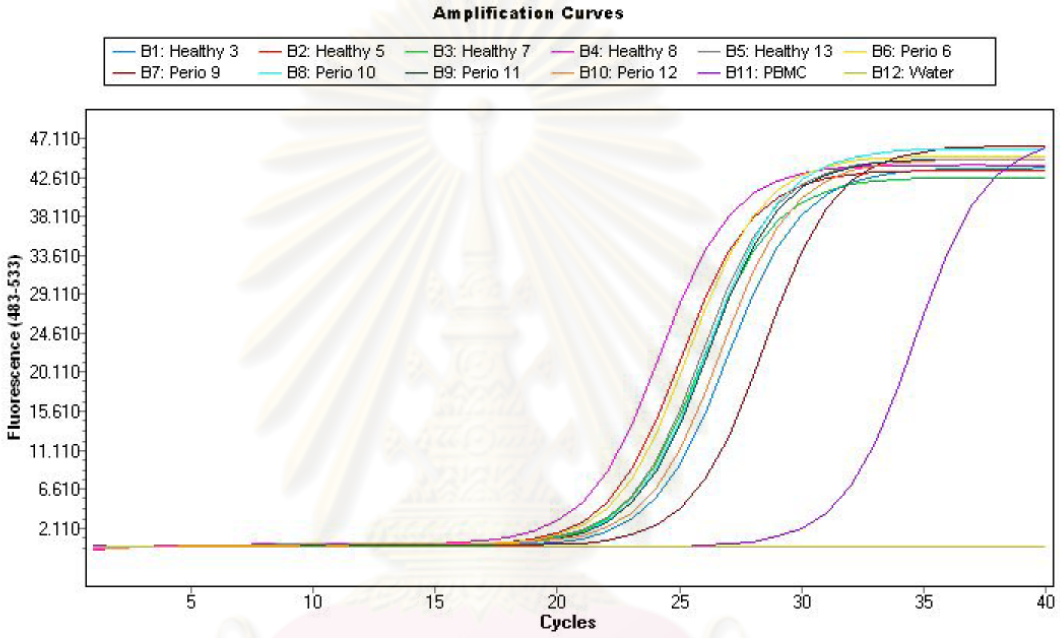
D5



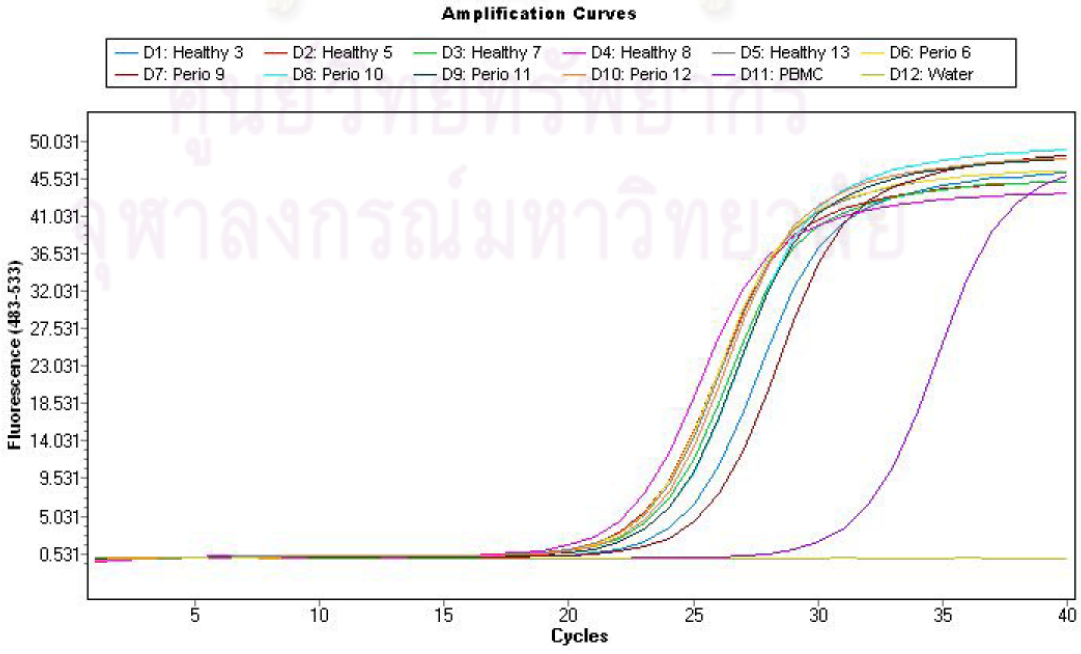
Appendix E : Amplification curves of amplified samples with cDNA derived from (E1) MxA, (E2) PKR, (E3) OAS-1, (E4) SLPI, and (E5) IFN-α.

As a negative control, template DNA was replaced by PCR- grade water.

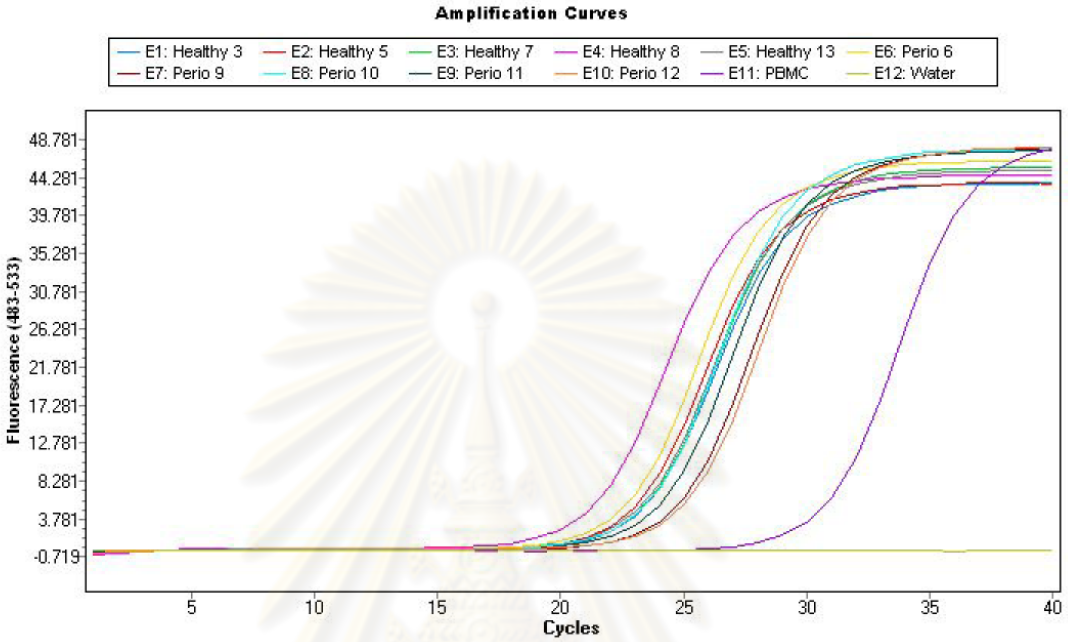
E1



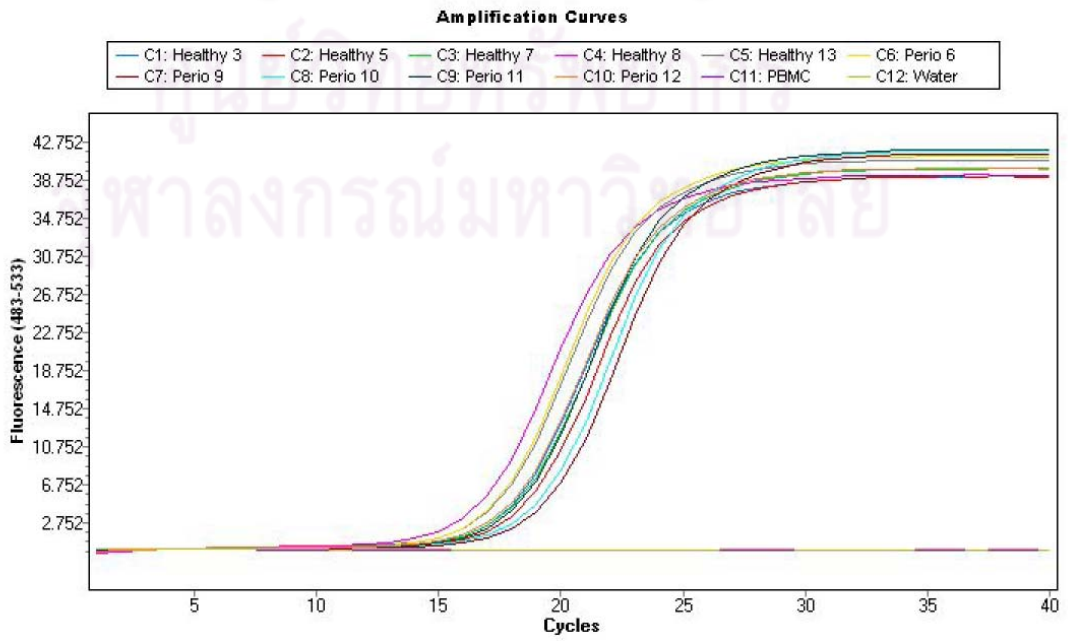
E2



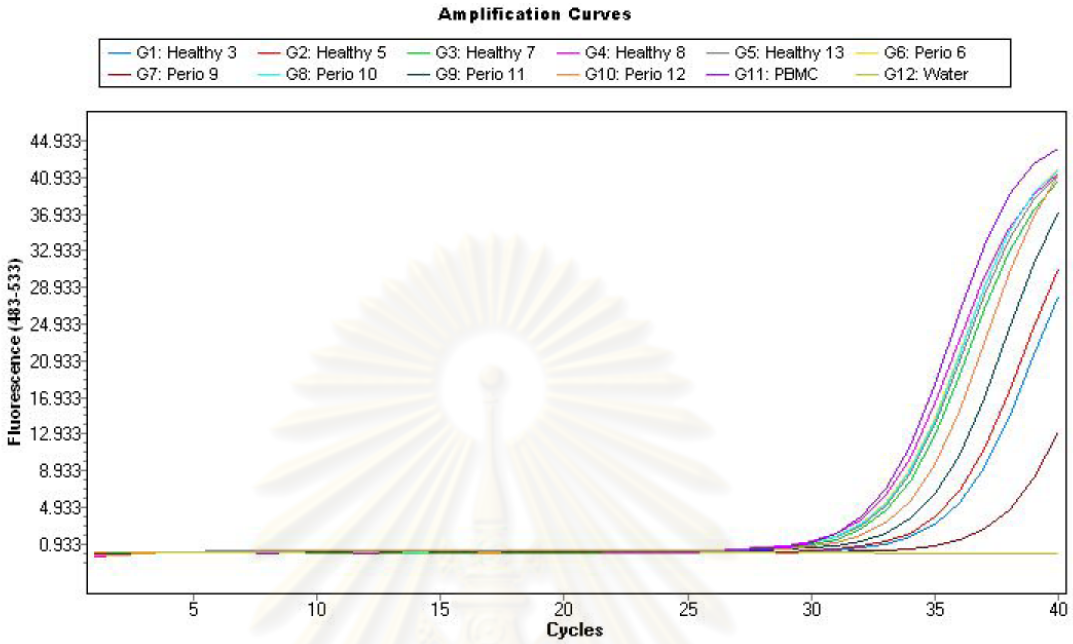
E3


Amplification Curves
E1: Healthy 3
E2: Healthy 5
E3: Healthy 7
E4: Healthy 8
E5: Healthy 13
E6: Perio 6
E7: Perio 9
E8: Perio 10
E9: Perio 11
E10: Perio 12
E11: PBMC
E12: Water
Fluorescence (483-533)
48.781
44.281
39.781
35.281
30.781
26.281
21.781
17.281
12.781
8.281
3.781
-0.719
5
10
15
20
25
30
35
40
Cycles

E4


Amplification Curves
C1: Healthy 3
C2: Healthy 5
C3: Healthy 7
C4: Healthy 8
C5: Healthy 13
C6: Perio 6
C7: Perio 9
C8: Perio 10
C9: Perio 11
C10: Perio 12
C11: PBMC
C12: Water
Fluorescence (483-533)
42.752
38.752
34.752
30.752
26.752
22.752
18.752
14.752
10.752
6.752
2.752
5
10
15
20
25
30
35
40
Cycles

E5



Appendix F : Threshold Cycle of GADPH, antiviral proteins and cytokine for each samples as well as negative and positive controls

No.	Group	Threshold Cycle (C_t)					
		GADPH	MxA	PKR	OAS-1	SLPI	IFN-α
1	Healthy	23.95	23.64	24.32	23.00	17.67	35.00
2	Healthy	24.62	21.74	22.65	22.65	18.15	35.00
3	Healthy	22.77	22.61	23.14	23.03	17.84	32.92
4	Healthy	21.14	20.86	21.94	20.99	16.34	32.29
5	Healthy	22.60	22.64	22.80	22.94	17.04	32.76
6	Periodontitis	21.53	22.03	22.71	22.31	16.95	32.66
7	Periodontitis	25.62	25.12	25.09	24.51	18.99	35.00
8	Periodontitis	23.57	22.85	23.63	23.15	18.72	32.70
9	Periodontitis	23.51	22.85	23.56	23.73	17.96	35.00
10	Periodontitis	23.50	23.42	23.02	24.75	17.66	34.01
	Water	-	-	-	-	-	-
	PBMC	32.91	31.23	31.47	30.51	29.97	32.16

Appendix G : Relative expression of mRNA of antiviral proteins and cytokine for each samples as well as negative and positive controls

No.	Group	Relative expression of mRNA of				
		MxA	PKR	OAS-1	SLPI	IFN-α
1	Healthy	1.239708	0.773782	1.931873	77.708473	0.000472
2	Healthy	7.361501	3.917681	3.917681	88.647006	0.000750
3	Healthy	1.117287	0.773782	0.835088	30.484416	0.000880
4	Healthy	1.214195	0.574349	1.109569	27.857618	0.000440
5	Healthy	0.972655	0.870551	0.790041	47.176615	0.000874
6	Periodontitis	0.707107	0.441351	0.582367	23.917588	0.000446
7	Periodontitis	1.414214	1.443929	2.158456	99.044160	0.001501
8	Periodontitis	1.647182	0.959264	1.337928	28.840015	0.001785
9	Periodontitis	1.580083	0.965936	0.858565	46.850742	0.000348
10	Periodontitis	1.057018	1.394744	0.420448	57.281605	0.000686
	Water	-	-	-	-	-
	PBMC	3.204280	2.713209	5.278032	7.674112	1.681793

Appendix H : Mann-Whitney U Test results of differences of mRNA expression of antiviral proteins and cytokine between periodontitis and healthy groups

Mann-Whitney U Test

Ranks

	group	N	Mean Rank	Sum of Ranks
MxA	Perio	5	5.60	28.00
	Healthy	5	5.40	27.00
	Total	10		
PKR	Perio	5	6.20	31.00
	Healthy	5	4.80	24.00
	Total	10		
OAS-1	Perio	5	4.80	24.00
	Healthy	5	6.20	31.00
	Total	10		
SLPI	Perio	5	5.20	26.00
	Healthy	5	5.80	29.00
	Total	10		
IFN-α	Perio	5	5.50	27.50
	Healthy	5	5.50	27.50
	Total	10		

Test Statistics (b)

	MxA	PKR	OAS-1	SLPI	IFN- α
Mann-Whitney U	12.000	9.000	9.000	11.000	12.500
Wilcoxon W	27.000	24.000	24.000	26.000	27.500
Z	-.104	-.733	-.731	-.313	.000
Asymp. Sig. (2-tailed)	.917	.463	.465	.754	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000(a)	.548(a)	.548(a)	.841(a)	1.000(a)

a Not corrected for ties.

b Grouping Variable: group

Appendix I : Descriptive data of the immunoreactive staining scores of MxA protein.

No.	Group	Immunostaining score			
		Site 1	Site 2	Site 3	Median
11	Healthy	3	3	3	**3**
12	Healthy	3	3	3	**3**
13	Healthy	3	2	3	**3**
14	Healthy	3	3	2	**3**
15	Healthy	2	3	3	**3**
16	Healthy	3	3	3	**3**
17	Healthy	3	3	3	**3**
18	Healthy	3	3	3	**3**
19	Healthy	3	3	3	**3**
20	Perio	3	3	2	**3**
21	Perio	2	2	2	**2**
22	Perio	1	2	1	**1**
23	Perio	2	3	2	**2**
24	Perio	3	3	3	**3**
25	Perio	2	2	3	**2**
26	Perio	3	3	2	**3**

Appendix J : Mann-Whitney U Test results of differences of immunostaining score of MxA proteins between periodontitis and healthy groups

Mann-Whitney U Test

Ranks

group		N	Mean Rank	Sum of Ranks
median	perio	7	5.93	41.50
	healthy	9	10.50	94.50
	Total	16		

Test Statistics (b)

	median
Mann-Whitney U	13.500
Wilcoxon W	41.500
Z	-2.516
Asymp. Sig. (2-tailed)	.012
Exact Sig. [2*(1-tailed Sig.)]	.055(a)

a Not corrected for ties.

b Grouping Variable: group

BIOGRAPHY

Miss Narisara Vanavit was born on 6th of April 1983 in Bangkok. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Chulalongkorn University in 2006, and became a governmental officer working as a dentist at Sangkha Hospital, Surin province in 2006 - 2008. She studied in Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2008.