ANALYSIS OF MICRORNA DERIVED FROM SALIVA- AND PLASMA-EXOSOME AS POTENTIAL BIOMARKER(S) FOR CHRONIC PERIODONTITIS

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

BMPR2	bone morphogenic receptor II
bp	base pair
BOP	bleeding on probing
°C	degree celcius
CAL	clinical attachment loss
cDNA	complementary deoxyribonucleic acid
CP	chronic periodontitis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FC	fold change
FBS	fetal bovine serum
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GCF	gingival crevicular fluid
GI	gingival index
hsa-miR	human microRNA
hyb	hybridization
IL1R1	interleukin 1 receptor 1
min	minute
miRNAs	microRNAs
µl	microliter
ml	mililiter
mm	milimeter
mRNA	messenger ribonucleic acid

NFKB1	nuclear factor kappa B-cells 1
ng	nanogram
nm	nanometer
nt	nucleotide
PBS	phosphate buffered saline
PPD	oeriodontal pocket depth
Pre-miRNA	intermediate miRNA
Pri-miRNA	primary miRNA
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
RIN	RNA integrity number
RNA	ribonucleic acid
TLR4	toll-like receptor 4
UTR	untranslated region

ANALISIS MIKRORNA DIPEROLEHI DARIPADA EKSOSOM AIR LIUR DAN PLASMA SEBAGAI PENANDA-BIO YANG BERPOTENSI UNTUK PERIODONTITIS KRONIK

ABSTRAK

Periodontitis kronik (CP) adalah penyakit rongga mulut yang timbul akibat keradangan kronik tisu periodontal. Eksosom adalah vesikel yang diperkaya dengan mikroRNA (miRNA) tertentu, berpotensi memberikan tanda diagnostik khusus penyakit. miRNA telah terbukti memainkan peranan penting dalam pengaturan tindak balas imuno-radang; namun, fungsi miRNA eksosom air liur dan plasma dalam keradangan periodontal kronik masih belum jelas. Untuk menaksir nilai miRNA eksosom sebagai penanda-bio yang berpotensi untuk penyakit CP, ekspresi miRNA dalam dua set sampel diprofil dan diukur menggunakan pelantar mikro susuntertib miRNA Agilent. Daripada 2,549 miRNA yang dikaji 1,985 dikawal selia menurun secara signifikan dan 10 dikawal selia menaik secara dalam sampel air liur CP; manakala 33 miRNA dikawal selia menurun secara signifikan dalam sampel plasma CP; berbanding dengan sampel kawalan masing-masing. Ekspresi hsa-miR-5006-5p, hsa-miR-942-3p, hsa-miR-30e-3p dan hsa-miR-199a-3p telah divalidasi menggunakan kaedah kuantitatif transkripsi terbalik tindak balas rantaian polimerase (RT-qPCR). Keputusan RT-qPCR merekodkan replikasi hasil pemprofilan miRNA dengan perubahan signifikan, memperkuatkan potensi miRNA tersebut untuk dikembangkan menjadi penanda-bio untuk penyakit ini. Untuk kajian *in vitro*, sel fibroblas gingiva manusia (HGFs) diinduksi dengan lipopolisakarida (LPS) daripada *Porphyromonas gingivalis* untuk meniru keadaan keradangan; manakala miRNA-miRNA tiruan digunakan untuk transfeksi. hsa-miR-30e-3p tiruan ditransfeksi sama

ada sebelum atau selepas induksi LPS untuk menguji reaksi tiruan terhadap situasi keradangan. Tahap hsa-miR-30e-3p hanya dikawal selia menaik secara signifikasi setelah diinduksi, menunjukkan kemampuan miRNA tiruan untuk bertindak sebagai penyembuh, dan bukan sebagai perisai. Terakhir, hsa-miR-5006-5p dan hsa-miR-30e-3p tiruan ditransfeksi ke dalam sel HGFs dan ekspresi reseptor protein morfogenetik tulang jenis II (*BMPR2*), interleukin 1 reseptor 1 (*IL1R1*), faktor nuklear kappa B subunit 1 (*NFκB1*) dan *TLR4* dikuantifikasi. *BMPR2, IL1R1, NFκB1* dan *TLR4* didapati dikawal selia menurun secara signifikan pada kedua-dua jenis sel yang ditransfeksi (sama ada ditransfeksi dengan hsa-miR-5006-5p tiruan atau hsa-miR-30e-3p tiruan). Namun, hanya kawal selia menurun *BMPR2* pada sel yang ditransfeksi dengan hsa-miR-5006-5p tiruan selari dengan kesan ekstrinsik keradangan, menjadikan *BMPR2* sebagai gen sasaran langsung untuk hsa-miR-5006-5p. Sementara itu, kawal selia menurun *IL1R1, NFκB1* dan *TLR4* yang signifikasi dalam sel yang ditransfeksi-dengan-hsa-miR-30e-3p-tiruan selari dengan kesan berlawanan ekstrinsik keradangan, menjadikannya berpotensi sebagai gen sasaran langsung untuk hsa-miR-30e-3p. Walaupun kajian mekanisma yang meluas diperlukan di masa depan, namun, hasil yang dapat direplikasi bermula daripada pemprofilan miRNA, kepada RT-qPCR, kepada kajian *in vitro* (terutamanya untuk eksperimen-eksperimen transfeksi hsa-miR-30e-3p tiruan/ induksi LPS), sudah cukup untuk meyakinkan potensi tinggi miRNA-miRNA ini sebagai penanda-bio CP.

ANALYSIS OF MICRORNA DERIVED FROM SALIVA- AND PLASMA-EXOSOME AS POTENTIAL BIOMARKER(S) FOR CHRONIC PERIODONTITIS

ABSTRACT

Chronic periodontitis (CP) is an oral cavity disease arising from chronic inflammation of the periodontal tissues. Exosomes are lipid vesicles enriched in specific microRNAs (miRNAs), potentially providing a disease-specific diagnostic signature. miRNAs have been demonstrated to play an important role in regulating the immuno-inflammatory response; however, the function of salivary and plasma exosomes miRNAs in chronic periodontal inflammation has not been investigated. To assess the value of exosomal miRNAs as potential biomarkers for CP, miRNA expression were profiled using Agilent miRNA microarray platform. From 2,549 tested miRNAs, 1,985 were significantly down-regulated and 10 significantly up-regulated in CP saliva samples; whereas 33 miRNAs were significantly down-regulated in CP plasma samples; as compared to respective healthy samples. Expression of hsa-miR-5006-5p, hsa-miR-942-3p, hsa-miR-30e-3p and hsa-miR-199a-3p was validated using reverse transcription quantitative polymerase chain reaction (RT-qPCR) method. RT-qPCR results recorded significant replicable miRNAs profiling results, strengthening the potential of these miRNAs to be developed into biomarker(s) for the disease. For *in vitro* study, human gingival fibroblasts (HGFs) cells were induced with lipopolysaccharides (LPS) from *Porphyromonas gingivalis* to mimic inflammation condition, whereas the miRNA mimics were used for transfection. hsa-miR-30e-3p mimic was transfected either before or after induction of LPS to test the reaction of the mimic towards

inflammation condition. hsa-miR-30-3p level was only significantly up-regulated after the induction, resembling the ability of the mimic to act as healer rather than as a shield. Lastly, the hsa-miR-5006-5p and hsa-miR-30e-3p mimics were transfected into HGFs cells and expression of bone morphogenetic protein receptor type II (*BMPR2*), interleukin 1 receptor 1 (*IL1R1*), nuclear factor kappa B subunit 1 (*NFκB1*) and *TLR4* were quantified. The *BMPR2, IL1R1, NFκB1* and *TLR4* were found significantly down-regulated in both types of transfected cells (either transfected with hsa-miR-5006-5p-mimic or hsa-miR-30e-3p-mimic). Yet, only the significant down-regulation of *BMPR2* in transfected cells-with hsa-miR-5006-5p-mimic parallel with extrinsic inflammation effect, making *BMPR2* as the direct target gene for hsa-miR-5006-5p. Meanwhile, the significant down-regulation of *IL1R1*, *NFκB1* and *TLR4* in transfected cells-with hsa-miR-30e-3p-mimic parallel with opposite extrinsic inflammation effects, making them as the potential direct target genes for hsa-miR-30e-3p. Although extensive mechanism study is necessary in future, nevertheless, the replicable results from miRNAs profiling, RT-qPCR, and *in vitro* study (especially for transfection of hsa-miR-30e-3p/ LPS induction experiments), are enough to ensure the high potential of these miRNAs as biomarkers for CP.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Periodontitis is an inflammatory disease of periodontal tissues, caused by specific bacteria and can be characterised by gingival swelling, loss of alveolar bone and movement of the teeth. Among the available types, chronic periodontitis (CP) is recognised as the most frequent occurring form of periodontitis (Suzuki *et al.,* 2004; Pihlstrom *et al.*, 2005; Kato *et al.*, 2013; Papapanou *et al.,* 2018; Hartenbach *et al.*, 2020; Inchingolo *et al.,* 2020; Aziz *et al.,* 2021; Kwon *et al.,* 2021; Macías-Camacho *et al.*, 2021; Marouf *et al.*, 2021; Romandini *et al*., 2021).

Human saliva has many important functions which are not only for oral system but also for other body systems. Over the past few years, numerous research articles had been published describing several salivary components and their distribution, confirming the biochemical composition and physiology of the proteins existed in the salivary fluids (Schenkels *et al.*, 1995; Baumann *et al.,* 2016; Khurshid *et al.*, 2016; Chojnowska *et al.,* 2018; Sanguansermsri *et al.,* 2018; Hartenbach *et al.,* 2020; Dave *et al.,* 2021; Marotz *et al.,* 2021). Many researchers have demonstrated the presence of exosomes in human saliva and their usage in various types of disease detection and investigation (Ogawa *et al.*, 2011, Huang *et al.*, 2020, Zhou *et al.*, 2020, Imai *et al.*, 2021, Rani *et al*., 2021).

Human blood products i.e. plasma and serum are the trendy and well-accepted choice for clinical diagnosis (Geyer *et al.*, 2016). This due to facts that blood is circulating the whole human body, surrounding the tissues and organs, hence is supposed to have the closest access to by-products from diseased areas (e.g.

tumours). Changes in the concentrations of specific components in plasma have been associated with diseases processes, leading to well-accepted for clinical applications (Loo *et al.*, 2010). Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in plasma have been reported to be able in detecting breast and lung cancer (Laktionov *et al.*, 2004), or as promising biomarker for liver fibrosis in non-alcoholic fatty liver disease (Huang *et al*., 2021). A large portion of plasma proteome have showed diagnostic and prognostic values for differentiating chronic kidney disease (CKD) across all stages (Christensson *et al.*, 2018), or as promising biomarker in Alzheimer's disease (Simrén *et al.*, 2021). Whereas, exosomes in plasma had been used for detecting and investigating diseases such as schizophrenia (Tan *et al.*, 2021), cardiovascular risk progression and early kidney damage (Perez-Hernandez *et al.*, 2021), as well as for pediatric haematological malignancies (Damanti *et al.*, 2021).

Several contents that presence in both saliva and plasma, such as hormones (Martin *et al.*, 2018), nucleotides (Neumann *et al.*, 2017; Neto *et al.*, 2018), proteins (Goldman *et al.*, 2018), microRNAs (miRNAs) (Talotta *et al.*, 2019), and exosomes (Shi *et al.*, 2014; Rani *et al.*, 2019; Luo *et al.*, 2020) have been used for comparison as better source for biomarker development. For example, Martin *et al.* (2018) had correlated oxytoxin level from human cerebrospinal fluid (CSF) with oxytoxin level from saliva and plasma. From 50 critically ill patients samples tested (neurological and neurosurgical diseases), oxytoxin level from saliva showed modest to strong correlations with the oxytoxin level in CSF compartments. Whereas, the oxytoxin levels from plasma showed weak correlation as to the level in CSF. Hence, they concluded that oxytoxin level in saliva could be serve as potential biomarker reflecting brain oxytoxin activity (Martin *et al.*, 2018). On other hand, Talotta *et al.* (2019) has tested salivary- and plasma-miRNAs from 28 primary Sjögren's

syndrome (pSS) patients, and found that salivary hsa-miR-146a can represent as pSS marker. In addition, expression of salivary hsa-miR-17, hsa-miR-18a and hsa-miR-146b were recorded to be altered in pSS patients, and can be associated with worse ultrasound, anti-La/ SSB (autoantibodies that occur in approximately 50% of pSS patients) positivity, and European League Against Rheumatism Sjögren's Syndrome Patient Reported Index (ESSPRI) score, respectively (Talotta *et al.*, 2019).

Exosomes are small vesicles of 30-120 nm in diameter (Pan *et al.*, 1985; Huang *et al.*, 2020), secreted by all types of cultured cells and found in abundance in body fluids, including saliva (Zlotogorski-Hurvitz *et al.*, 2015; Kamal *et al.*, 2020), blood (Damanti *et al.*, 2021; Perez-Hernandez *et al.*, 2021; Tan *et al.*, 2021), urine (Hiltbrunner *et al.*, 2020; Yang *et al.*, 2020; Perez-Hernandez *et al.*, 2021), ascites (Yun *et al.*, 2019; Cai *et al.*, 2021), amniotic fluid (Bellio *et al.*, 2020; Tavansefat *et al.*, 2020) and cultured medium of cell cultures (Huang *et al.*, 2020; Ivica *et al.*, 2020) including reticulocytes (Martín-Jaular *et al.*, 2017; Díaz-Varela *et al.*, 2018); cytotoxic T lymphocytes (Ogawa *et al.*, 2011; Chen *et al.*, 2019); B lymphocytes (Raposa *et al.*, 1996; Escola *et al.*, 1998; Calvo *et al.*, 2020); dendritic cells (Tambyah *et al.*, 2015; Cao *et al.*, 2021) and neoplastic intestinal epithelial cells (Thery *et al.*, 2001; Scavo *et al.*, 2020). Exosomes serve as carriers for various microRNAs (miRNAs). These small vesicles play an important part in intercellular communication, both in the local environment and systemically, facilitating the transfer of proteins, cytokines as well as miRNA between cells (Valadi *et al.*, 2007; Yang *et al.*, 2011; Gallo *et al.*, 2012; Hergenreider *et al.*, 2012). Review article had been published comparing either non-exosomal or exosomal miRNAs are more valid as biomarkers. From 3,558 articles selected using the keyword "miRNA AND circulating", 32 articles were further grouped together in comparing non-exosomal

and exosomal miRNAs efficiency as biomarkers. From this 32 articles, 18 articles chose exosomes; one article chose extracellular vesicles; one article chose multivesicles; three articles chose Argonaute (Ago-2) complexes; one article chose cell-free; seven articles chose unfraction; while one article showed similar expression between whole serum versus exosomes; as the best origin for miRNAs used in biomarker studies (Nik Mohamed Kamal & Shahidan, 2020).

miRNAs are non-protein-coding RNA molecules consist of 20-25 nucleotides (Lee *et al.*, 2011; Wu *et al.*, 2021), acting as post-transcriptional regulators via binding to the 3' untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs) and usually resulting in gene silence. They have negative regulation of gene expression, particularly by degrading target mRNA or inhibit the translation of protein product (Lee *et al.*, 2011) and have been well characterised to play roles in cell growth (Coppola *et al.*, 2021; Kong *et al.*, 2021), differentiation (Kornfeld *et al.*, 2021; Wang *et al.*, 2021a), apoptosis (Sun *et al*., 2020; Wang *et al*., 2021b), host-pathogen interactions (Acuña *et al.*, 2020; Wang *et al*., 2020a; Afrasiabi *et al*., 2021), stress responses (Andolina & Di Segni Matteo, 2017; Luo *et al.*, 2021; Sharma *et al.*, 2021) and immune function (Wang *et al.*, 2020b; Zang *et al.*, 2019). miRNAs have been proposed as excellent salivary biomarker candidates due to their ease of isolation and identification through quantitative PCR (Michael *et al.*, 2010; Dlugash & Schultheiss, 2021). Research related to salivary miRNA analysis has been done to understand the pathophysiological reasons for the alterations in saliva production as well as the pathogenesis of salivary gland tumours (Liu *et al.*, 2012). miRNAs have been demonstrated to play an important role in the regulation of immuno-inflammatory response (Xie *et al*., 2011; Wang *et al.*, 2021c).

Recently, there were few studies demonstrated that miRNAs can be found encapsulated in microvesicles such as exosomes (Michael *et al.*, 2010; Wan Nazatul Shima, 2012; Rani *et al.*, 2021; Tan *et al.*, 2021) and that those miRNAs exist primarily inside those salivary exosomes rather than in the free form (Gallo *et al.*, 2012). Indeed, the concentration of miRNAs in the exosomes was very high (Zeng, 2006). Previous study has compared miRNA profiles of human periodontal disease and healthy gingival tissues among periodontitis subjects and healthy subjects. They found that twelve selected inflammatory-related miRNAs, hsa-miR-126*, hsa-miR-20a, hsa-miR-142-3p, hsa-miR-19a, hsa-let-7f, hsa-miR-203, hsa-miR-17, hsa-miR-223, hsa-miR-146b, hsa-miR-146a, hsa-miR-155, and hsa-miR-205 showed comparable expression levels by microarray and reverse transcription quantitative real-time PCR (RT-qPCR) analyses. The putative inflammation targets of these miRNAs were predicted, and three out of them (hsa-miRNA-146a, hsa-miRNA-146b, and hsa-miRNA-155) showed significant differences between the inflamed and healthy gingival tissues. This finding indicates a probable relationship between miRNA and periodontal inflammation (Xie *et al.*, 2011). Meanwhile, Ogata *et al.* (2014) has profiled miRNA expression in inflamed and noninflamed gingival tissues from Japanese dental patients; and confirmed the findings via real-time PCR (qPCR). They found that three most overexpressed (by >2.72-fold) miRNAs were hsa-miR-150, hsa-miR-223, and hsa-miR-200b, and the three most underexpressed (by <0.39-fold) miRNAs were hsa-miR-379, hsa-miR-199a-5p, and hsa-miR-214. In hybrid models like GO hybrid (IPA) analysis, hsa-miR-150, hsa-miR-223, and hsa-miR-200b were associated with inflammatory disease, organism injury, abnormalities, urological disease, and cancer. This finding indicates certain miRNA associated with CP in Japanese patients (Ogata *et al.*, 2014).

The expression of miRNAs in periodontal tissues has been elucidated (Ogata *et al.*, 2014), however, their potential function in the context of miRNA analysis of exosome-derived saliva and plasma in chronic periodontal diseases are largely unexplored. Therefore, this study will mainly analyse the occurrence of saliva- and plasma-derived exosome miRNAs in healthy and CP and subsequently validate their *in silico*-predicted targets by examining mRNA profiles in the same specimens. The finding in the concomitant study of saliva- and plasma-derived exosome miRNA and their miRNA expression in periodontal disease would provide new insights into the *in vitro* gene-regulatory function of miRNAs in human with periodontitis.

1.2 Problem statement

According to National Oral health Surveys by Ministry of Health Malaysia, the periodontal disease prevalence in adults had recorded declination between year 1990 (92.8%) to year 2000 (90.25), but experience a sharp rise in year 2010 (94.0%) (National Oral health Surveys, Ministry of Health Malaysia). The rising of prevalence had increased the burden in dental health care. Using this data, Dom and collegues reported that, with the passing years, there were shiftment of patients with only having dental calculus to more complex conditions involving the development of shallow and deep pockets (Mohd-Dom *et al.*, 2013). Poor oral hygiene would leads to bacteria invasions in periodotium or surrounding tissues, bacteria adhesion and co-aggregation, followed by plaque formation. Calcification of plaque would build-up calculus or tartar. Production of bacterial enzymes, exotoxins or endotoxins affecting the pathological of surrounding teeth tissue, creating inflammation condition (gingivitis). Untreated gingivitis could end-up to form chronic periodontitis disease, characterised by development of periodontal pocket, as a result

from degeneration of collagen, periodontal ligament and alveolar bone. At severe stage, periodontal tissue destruction continues, which might lead to further alveolar bone resorption and tooh mobility as well as tooth loss (Figure 1.1). Periodontitis is recorded to be the major cause of teeth loss in adults, directly affecting masticatory function, nutrition and aestetic appearance, which linked to a person's quality of life (Jain *et al.,* 2008; Wong *et al.,* 2020). Hence, this highlighted the importance of detecting the periodontitis condition as early as possible to avoid the loss of healthy teeth due to the state of periodontal disease. To date, periodontitis condition is always clinically diagnosed, based on clinical parameters such as the charting of probing depth (measurement using probe to estimate the depth of formed sulcus or pocket between gum and teeth, i.e. the distance between the base of the pocket and gingival margin (Listgarten, 1980; Newman *et al.,* 2012)), attachment loss (measurement using probe, of the distance between the base of the pocket and a fixed point on th crown such as the cementoenamel junction), bleeding on probing (the inspection on whether or not the insertion of the probe to the bottom of the pocket would elicit bleeding) and radiographic findings (alveolar bone loss estimation by measuring the difference between the physiological bone level and the height of remaining bone from radiographic image captured using x-ray technique (Newman *et al.,* 2012) (Figure 1.2). These diagnosis required specific skills, and sometimes cause variation in results, due to individual interpretation. The diagnosis of periodontitis condition at early stage on the basis of clinical diagnostic criteria is quite challenging. Accordingly, several trials needed to fully diagnose the disease at

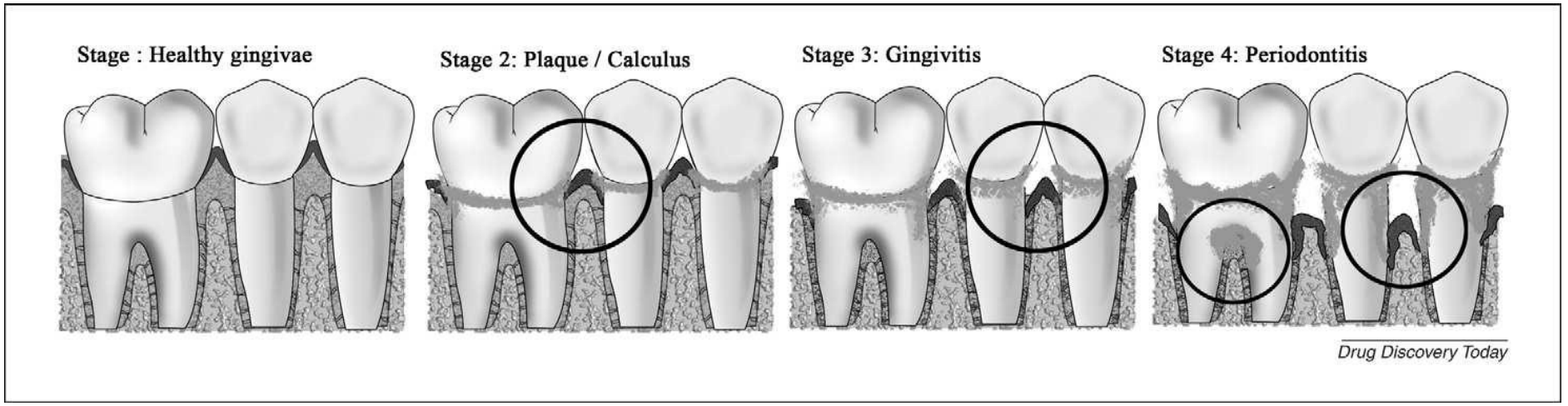

Figure 1.1 Healthy gingivae versus pathological periodontitis. Stage 1: healthy gum tissue (gingiva); stage 2: plaque formation due to bacterial invasion; stage 3: bacterial toxins irritate gums and trigger host-mediated responses that lead to gingivitis; stage 4: destruction of gingiva and bone that support the tooth leading to periodontitis (Jain *et al.*, 2008). The permission of using these figures are as in Appendix 1.

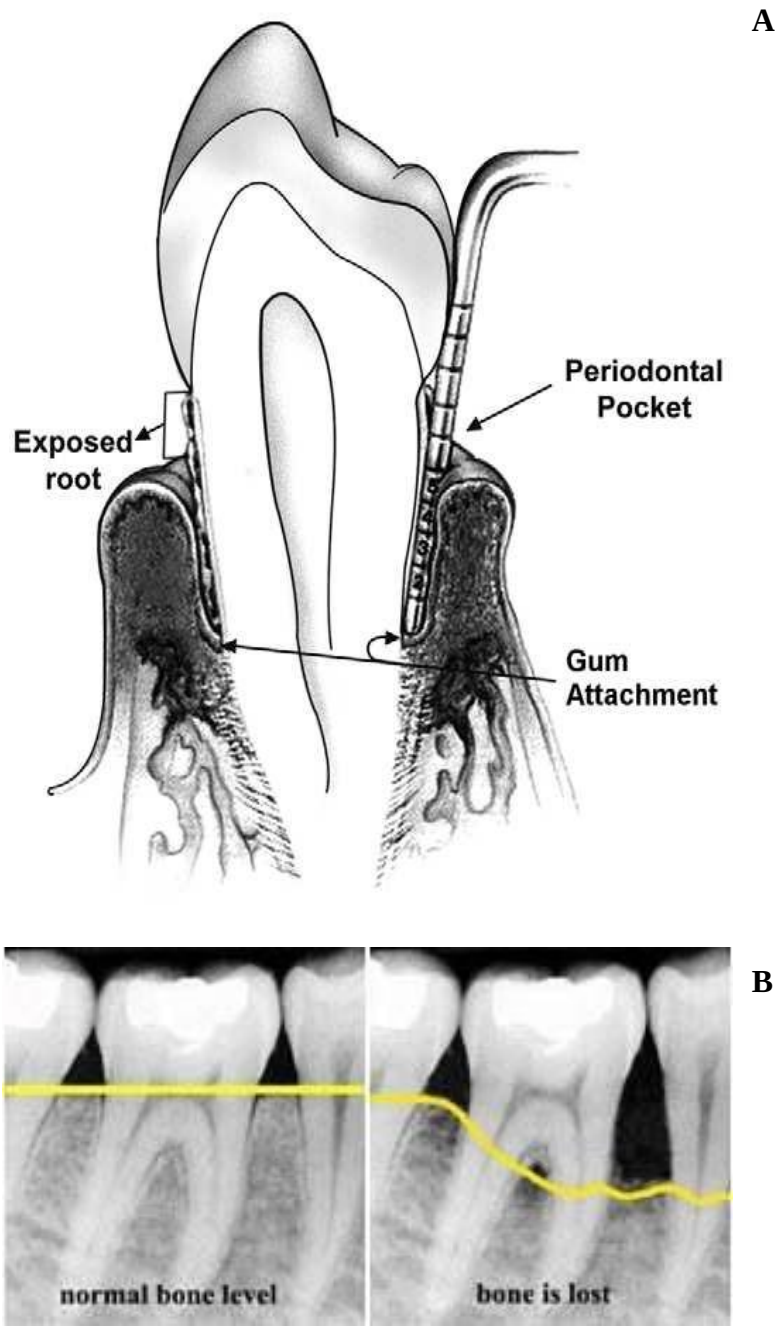

Figure 1.2 Periodontal pocket depth, the gum attachment, probe used for measurement (Jain *et al.*, 2008) are shown as in **A,** and radiographic evidence of alveolar bone loss is shown as **B** (https://www.firstimpressionsdds.com/blog/periodontaldisease/). The permission of using these figures are as in Appendix 1.

early stage (Kim and Kim, 2021). The problem rely on the state of the disease. Periodontal disease rarely causing painful condition to the patient, hence, leading to ignorance. By the time patients reach out to the dentist, their periodontitis conditions would already worsen (Newman *et al.*, 2012). Hence, having biomarker(s) that could detect early stage of periodontitis condition would be a great help for the clinical diagnosis, prognosis and management.

1.3 Hypothesis

Certain exosomal miRNAs in saliva and plasma are involved in regulation of the inflammatory gene expression in CP, which enabling them to be used as biomarker(s) for detecting CP disease.

1.4 Objectives

General

To explore miRNA expression of saliva- and plasma-exosomes in CP subjects.

Specific:

1. To profile the miRNAs in saliva and plasma exosomes of healthy subjects and CP subjects using miRNA microarray.
2. To compare the differential expression pattern of miRNAs in saliva and plasma exosomes of healthy subjects and CP subjects.
3. To validate the differential miRNA expression in salivary exosomes using RT-qPCR.
4. To predict the putative inflammatory-related target genes for the validated miRNAs using TargetScan prediction.

5. To choose the key target genes that could serve as a potential target for chronic inflammation suppressor by overlapping the predicted target genes by TargetScan with the target genes that have been recorded from previous related studies. .

6. To investigate the inflammatory target genes expression of putative exosomal miRNAs using mimics tranfection and LPS induction methods.

CHAPTER 2

LITERATURE REVIEW

2.1 Periodontitis

Periodontitis is an inflammatory disease characterised by tissue destruction around dental roots and alveolar bone, resulting in increase of probing depth formation, recession, or both. Factors that can contribute to the progression of periodontitis disease are such as systemic alterations (e.g. diabetes, cardiovascular diseases, and osteoporosis) and biofilm formation in the gingival sulcus (Newman *et al.*, 2015; da Silva *et al.*, 2017).

The current management for periodontal disease includes a proper diagnosis of the state of the disease (by using clinical parameters assessment), plan for eliminating the causes (by scaling: to remove the build-up plaque), and plan for reducing modifiable risk factors of the disease (e.g. advicing the patients to restrict themselves from smoking and to have a good oral hygiene). If the disease is not at severe stage, several set of reveiws, scaling and root planing would be sufficient for treatment. If one has sites with active periodontitis at periodontal re-evaluation, a contemporary regenerative or traditional resective surgical therapy can be utilised. Thereafter, periodontal maintenance therapy at a regular interval and long-term follow-ups are also crucial to the success of the treatment and long-term retention of teeth (Kwon *et al.*, 2021).

According to the published paper 2018, by Papapanou and colleagues, with the title “Periodontitis: Consensus report of workgroup 2 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions”, a new periodontitis classification scheme had been adopted as a revision of internationally

accepted classification dated in 1999 (Armitage, 1999). The previous classification of "chronic" and "aggressive" periodontitis are now grouped into a single category as "periodontitis", and further divided using the "staging and grading" system. The "staging" system is characterised based on the severity of the disease at presentation and the complexity of disease management to be engaged (Table 2.1). Meanwhile, the "grading" system is characterised by additional information gathered regarding the biological features of the disease (history-based analysis to rate the periodontitis progression), assessment of the risk for further progression, analysis of the possibility of poor outcomes of proposed treatment and assessment of the risk that disease and the treatment might negatively impact the general health of the patient (Table 2.2). There are four stages (stage I, stage II, stage III and stage IV); and three grades have been established (grade A: slow rate of progression; grade B: moderate rate of progression; and grade C: rapid rate of progression) (Tables 2.1 and 2.2) (Papapanou *et al.*, 2018). Nevertheless, as this study was started in 2017, earlier than the publication of a new classification scheme, hence, this study followed the original accepted classification scheme outlined by the AAP Workshop for the International Classification of Periodontal Diseases in 1999. This classification divided periodontitis into three general clinical manifestations: chronic periodontitis (CP), aggressive periodontitis, and periodontitis as a manifestation of a systemic disease (Armitage, 1999; Newman *et al.*, 2015).

Table 2.1 The staging system.Table taken from Papapanou *et al.*, 2018 showed the classification of periodontitis based on stages, which defined by severity, complexity & extent and distribution.

Periodontitis stage		**Stage I**	**Stage II**	**Stage III**	**Stage IV**
Severity	**Interdental CAL at site of greatest loss**	1 to 2 mm	3 to 4 mm	≥ 5 mm	≥ 5 mm
	Radiographic bone loss	Coronal third (< 15%)	Coronal third (15% to 33%)	Extending to middle or apical third of the root	Extending to middle or apical third of the root
	Tooth loss	No tooth loss due to periodontitis		Tooth loss due to periodontitis of ≤ 4 teeth	Tooth loss due to periodontitis of ≥ 5 teeth
Complexity	**Local**	-Maximum probing depth ≤ 4 mm -Mostly horizontal bone loss	-Mazimum probing depth ≤ 5 mm -Mostly horizontal bone loss	<u>In addition to stage II complexity:</u> -Probing depth ≥ 6 mm -Vertical bone loss ≥ 3 mm -Furcation involvement Class II or III -Moderate ridge defect	<u>In addition to stage III complexity:</u> Need for complex rehabilitation due to: -Masticatory dysfunction -Secondary occlusal trauma -Severe ridge defect -Bite collapse, drifting, flaring -Less than 20 remaining teeth (10 opposing pairs)
Extend and distribution	**Add stage as descriptor**	For each stage, describe extent as localized (< 30% of teeth involved), generalized, or molar/ incisor pattern			

Table 2.2 The grading system. Table taken from Papapanou *et al.*, 2018 showed the classification of periodontitis based on grades that reflect biologic features of the disease (i.e. direct and indirect evidences, as well as risks factors).

Periodontitis grade			**Grade A: Slow rate of progression**	**Grade B: Moderate of progression**	**Grade C: Rapid rate of progression**
Primary criteria	Direct evidence of progression	Longitudinal data (radiographic bone loss or CAL)	Evidence of no loss over 5 years	< 2 mm over 5 years	≥ 2 mm over 5 years
	Indirect evidence of progression	% bone loss/ age	< 0.25	0.25 to 1.0	> 1.0
		Case phenotype	Heavy biofilm depositis with low levels of destruction	Destruction commensurate with biofilm deposits	Destruction exceeds expectation given biofilm deposits; specific clinical patterns suggestive of periods of rapid progression and/ or early onset disease (e.g. molar/ incisor pattern; lack of expected response to standard bacterial control therapies)
Grade modifiers	Risk factors	Smoking	Non-smoker	Smoker < 10 cigarattes/ day	Smoker ≥ 10 cigarattes/ day
		Diabetes	Normol glcemic/ no diagnosis of diabetes	HbA1c < 7.0% in patients with diabetes	HbA1c ≥ 7.0% in patients with diabetes

2.1.1 Chronic periodontitis (CP)

Chronic periodontitis (CP) is previously known as “adult periodontitis” or “chronic adult periodontitis” as it is more common to be diagnosed in people age 35 years old and above. However, with new findings and technology, this type of periodontitis can also be found in children and adolescents, making changing the name into universal “chronic periodontitis” is much more suitable (Newman *et al.,* 2012). CP is known to be a slow progressive disease and not causing the painful symptom, resulting in diagnosis in early-stage to be hard (Newman *et al.,* 2012; Kim and Kim, 2021). However, the present factors such as diabetes (Preshaw and Bisset, 2019; Bakari *et al.,* 2021; Miller *et al.,* 2021), smoking (Amaranath *et al.,* 2020; Duarte *et al.,* 2021), stress (Castro *et al.,* 2020; Petit *et al.,* 2021), and chronic diseases (Cardoso *et al.,* 2018; Kaushal *et al.,* 2019) that could enhance plaque accumulation could turn on the aggressiveness of the disease (Newman *et al.,* 2012).

General characteristics for untreated CP patient are supragingival and subgingival plaque accumulation, gingival inflammation, loss of periodontal attachment, loss of alveolar bone, and occasional suppuration. Meanwhile, in treated patient with poor oral hygiene, the characteristic would be slight to moderate swollen of the gingival, exhibition of alteration in gingival colour ranging from pale red to magenta, loss of gingival stippling, and changes in surface topography may include blunted or rolled gingival margins and flattened or cratered papillae. On other hand, for patient performing regular home care measures, the changes of gingival colour, contour and consistency that frequently associated with gingival inflammation may not be visible on inspection, and inflammation may be detected only as bleeding with a periodontal probe (Newman *et al.,* 2012).

CP can be further divided into three groups, which are slight (mild), moderate and severe, based on the severity of the destruction of periodontium (Newman *et al.*, 2012; Natto *et al.*, 2018; Kim and Kim, 2021). Slight (mild) periodontitis is clinically diagnosed to whom with occurrence of no more than 1 to 2 mm of clinical attachment loss. Moderate periodontitis is diagnosed to those with periodontal destruction of 3 to 4 mm of clinical attachment loss. Whereas, severe periodontitis is diagnosed to those with clinical attachment loss of 5 mm or more (Newman *et al.*, 2012; Natto *et al.*, 2018).

2.1.2 Bacteria associated with periodontal disease

Porphyromonas gingivalis (*P. gingivalis*) (Damgaard *et al.*, 2019; Ardila and Bedoya-García, 2020; Zhang *et al.*, 2021) along with *Tannerella forsythia (T. forsythia)*, *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) (Lauritano *et al.*, 2016; Rodrigues *et al.*, 2018; Ardila and Bedoya-García, 2020), *Fusobacterium nucleatum* (*F. nucleatum*) (Lauritano *et al.*, 2016; Rodrigues *et al.*, 2018) and *Prevotella intermedia* (*P. intermedia*) (Deng *et al.*, 2017; Dorneanu *et al.*, 2018) are bacteria species that had been studied for along time for their association with periodontal disease.

P. gingivalis, an anaerobic, non-motile, Gram-negative, short rod/ slightly elongated coccus bacterium, has been reported by several studies to abundantly populated in sites with periodontitis and in lesser to a non-detectable presence in sites with healthy gingival or plaque-induced gingivitis. The elimination of *P. gingivalis* had been demonstrated to successful clinical outcomes for treating periodontitis, while the persistence of the bacteria associated to reoccurrence of the disease (Ximenez-Fyvie *et al.*, 2000a; Ximenez-Fyvie *et al.*, 2000b; Wilson and Kornman,

2003; Mysak *et al.*, 2014; Lauritano *et al.*, 2016; Damgaard *et al.*,2019; Ardila & Bedoya-García, 2020; Zhang *et al.*, 2021). *P. gingivalis* had been recorded to have virulence factors such as true collagenase, endotoxin, immunoglobulin A (IgA) and other proteases which enable the microorganism to induce bone resorption, destruct connective tissues, induce production of various types of cytokines as well as inhibit host protective mechanisms (Papapanou *et al.*, 1994; Mysak *et al.*, 2014).

Lipoplysaccharides (LPS) are large molecules with a composition of lipid A and polysaccharides component that can be found in the outer membrane of Gram-negative bacteria such as *P. gingivalis*. LPS acts as endotoxins, and it can be recognised by the human innate immune system via the binding with toll-like receptors (TLRs). TLRs, the cell surface receptors or also known as pattern-recognition receptors (PRRs), recognise the microbe-associated molecular patterns (MAMPs), which are conserved molecular structures located on diverse types of pathogens. TLR4 able to recognise LPS from Gram-negative bacteria, lead to the binding process and forming complex cell surface molecules of cluster of differentiation 14- toll-like receptor 4- myeloid differentiation factor 2 (CD14-TLR4-MD2). The CD14-TLR4-MD2 complex triggers several constitutive intracellular events to increase the production of proinflammatory mediators (cytokines) and differentiation of immune cells to develop immune responses against the particular invading pathogens. LPS from *P. gingivalis* known to be atypical and can be recognized by both TLR2 and TLR4 (Preshaw and Taylor, 2012; Lin *et al.*, 2017; Nativel *et al.*, 2017; Zhang *et al.*, 2018b). Apart from the described set of PPR-MAMP-bacteria (TLR2/4-LPS-*P. gingivalis*) above, other examples of PPR-MAMPS-bacteria set that involved in the development of periodontal disease are summarized as in Table 2.3.

Table 2.3 List of examples for pattern recognizing receptors (PRRs), microbe-associated molecular patterns (MAMPs) and the involved bacteria that associated with the development of periodontal disease (Newman *et al.*, 2012).

PRR	MAMP	Periodontal bacteria
TLR2	Lipoproteins	*T. forsythia*
	Atypical LPS	*P. gingivalis, Capnocytophaga ochracea* (*C. ochracea*)
	Fimbriae	*P. gimgivalis*
	Non-endotoxin glycoprotein	*P. intermedia*
	Whole bacteria	*Treponema denticola*
	Cell surface BspA protein	*T. forsythia*
TLR4	LPS	*P. gingivalis, C. ochracea*
	Heatshock protein (HSP)-60	*P. gingivalis*
	Atypical LPS	*P. gingivalis*
TLR9	CpG-containing DNA	*A. actinomycetemcomitans, P. gingivalis*

2.1.3 Selected inflammatory mediators affecting periodontal tissues

The untreated long-term presence of subgingival biofilm (plaque located below the gingival margin, between the tooth and gingival pocket epithelium) would lead to "bystander damage" condition; where the host immune response leads to the tissue damage, hence presenting the clinical symptoms of periodontitis such as the deepening of pocket depth and the increase in clinical attachment loss (Preshaw and Taylor, 2012; Kim and Kim, 2021). The excessive or dysregulation of several inflammatory mediators either from the group of cytokines, prostaglandins or matrix metalloproteinases are held responsible for deriving the tissue damage occasion (Preshaw and Taylor, 2012).

Cytokines are soluble proteins produced by cells in response to certain stimuli. Cytokines can be further divided according to the type of stimulation: (i) autocrine stimulation: the produced cytokines work on the parent cells (Clemens, 1991; Chong *et al.*, 2020; Sid-Otmane *et al.*, 2020); (ii) paracrine stimulation: the produced cytokines work on nearby cells (Clemens, 1991; Sid-Otmane *et al.*, 2020; Heo *et al.*, 2021); (iii) similar to an endocrine stimulation by classical hormones: the produced cytokines are released into the bloodstream or other body fluids and interact with cells that elsewhere in the body (Clemens, 1991; Sid-Otmane *et al.*, 2020). Among the well-studied cytokines, interleukin 1β (IL1β) (Aleksandrowicz *et al*., 2021; Pani *et al.*, 2021) and tumour necrosis factor alpha (TNFα) (Jakovljevic *et al*., 2020; Aleksandrowicz *et al*., 2021) are related to periodontal disease. IL1β and TNFα were reported to initiate, regulate and maintain the periodontium innate immune responses which lead to vascular changes and migration of effector cells to fight the presence of bacteria (Preshaw and Taylor, 2012; Jakovljevic *et al*., 2020; Aleksandrowicz *et al*., 2021; Pani *et al.*, 2021).

Prostaglandins (PGs), a group of lipid compounds derived from polysaturated fatty acid (arachidonic acid) in the plasma membrane of cells (Preshaw and Taylor, 2012). Cyclooxygenases-1 and -2 (COX1 and COX2) metabolises the arachidonic acids and generate prostanoids (the combination of PGs, thromboxanes and prostacyclins) (Synthases, 2004). Up-regulation of COX2 by IL1β/ TNFα/ LPS will increase the production of prostaglandin E2 (PGE_2) (Sugimoto and Narumiya, 2007), which induce the production of matrix metalloproteinases (MMPs), and lead to osteoclastic bone resorption and tissue damage (Preshaw and Taylor, 2012).

MMPs or matrixins, are zinc-dependent proteolytic enzymes that degrade extracellular matrix molecules e.g. collagen, gelatin and elastin. MMPs can be further divided into six categories: collagenases, gelatinases/ type IV collagenases, stromelysins, matrilysins, membrane-type metalloproteinases and others (MMPs with no group designation) (Zitka *et al.*, 2010; Singh *et al.*, 2015). MMPs are secreted in latent form and only activated by proteolytic cleavage done by proteases such as cathepsin G. MMPs can be inhibited by either proteinase inhibitors which have anti-inflammatory properties. Some of MMPs inhibitors are: (i) the glycoprotein α_1-antitrypsin and α_2-macroglobulin: which can be found in serum; (ii) tissue inhibitors of metalloproteinases (TIMPs): which can be found in tissues; and (iii) tetracycline class of antibiotics (Nagase & Woessner, 1999; Preshaw & Taylor, 2012; Fields, 2015).

2.1.3(a) Bone morphogenetic protein receptor type 2 (BMPR2)

Bone morphogenetic proteins (BMPs) are grouped under transforming growth factor β and growth factors family of cytokines (Pickup *et al.*, 2015). BMPs are known to have roles in inducing the formation of bone and cartilage; regulating the

development of tooth, kidney, skin, hair, muscle, haematopoietic and neuron; as well as maintaining the iron metabolism and vascular homeostasis *in vivo*. By undergo processing, and facilitation of co-receptors, the secreted BMPs ligand would bind to their cognate serine/ threonine kinase receptors, which mediates the transduction signals through either Smad or non-Smad signalling pathways (Miyazono *et al.*, 2010; Harper *et al.*, 2016; Kim *et al.*, 2017).

Type I receptors for TGFβ-family in mammals are known as activin receptor-kinases (ALKs). There are seven ALKs that had been identified, which further categorised into three groups: (i) ALK1 group (consists of ALK1 and ALK2); (ii) BMPRI group (consists of ALK3 and ALK6); and lastly, (iii) TβRI group (consists of ALK4, ALK5 and ALK7). The ALK1 and BMPRI groups are both responsible for activating the Smad 1/5/8, while the TβRI activates Smad 2/3, to transducer intercellular signals (Kawabata *et al.*, 1998; Miyazono *et al.*, 2010; Orriols *et al.*, 2017).

BMPs type II receptors also can be divided into three groups: (i) the BMPRII/ BMPR2, which only can be used by BMPs; while both (ii) the ActRII; and (iii) the ActRIIB, can be used by BMPs, as well as by activins and myostatins (Yu *et al.*, 2005; Miyazono *et al.*, 2010; Orriols *et al.*, 2017).

Several published studies had recorded the effects of down-regulation or loss of BMPR2: (i) mutation in various regions of BMPR2, although cannot innate the disease process, somehow, contribute to higher chances in getting primary pulmonary arterial hypertension (PAH) (mutations found in 70% of patients with PAH, and 10-40% in patients with idiopathic PAH (Oh & Li, 1997; Lane *et al.*, 2000); (ii) down-regulation of BMPR2 promotes the excessive inflammatory response (e.g. increase the level of interleukin 6 (IL6) and interleukin 8 (IL8)) both

in vitro and *in vivo* that induced with LPS (Paulin *et al.*, 2012; Perros and Bonnet, 2015); (iii) absence of stromal BMPR2 expression lead to increase of tumour metastasis and inflammatory cell infiltration in human breast cancer patients (Pickup *et al.*, 2015); while (iv) loss of BMPR2 led to decrease in JNK (c-Jun-N-terminal protein kinases) signaling in endothelial cells (ECs), which enhance the BMP9-induced mineralization (vascular calcification) that associated with prevalent feature in cardiovascular diseases with high risk of mortality rate (Sanchez-Duffhues *et al.*, 2019). Hence, these studies showed that the expression of BMPR2 is negatively related to inflammation level: the decrease of BMPR2 would portray the increase of inflammation condition, while the increase of BMPR2 would portray the decrease in the inflammation condition.

2.1.3(b) Interleukin 1 receptor type 1 (IL1R1)

Interleukin (IL) and interferon γ are two types of lymphokines- the cytokines group that produced by or act on cells related to immune systems (Clemens, 1991). IL1 family consists of: (i) IL1 (α and β), IL33, IL8 and IL36 (α, β, γ) that are ligands with agonist activity; (ii) IL1Ra, IL38 and IL36Ra are receptor agonists; while (iii) IL37 is an anti-inflammatory cytokine (Garlanda *et al.*, 2013; Chakraborty *et al.*, 2020). There are two types of interleukin 1 receptors (IL1R), which are: IL1R1 and IL1R2. Interaction of IL1R1 with IL1 receptor accessory protein (IL1RAP) will lead to the development of transmembrane signalling complex, which initiates IL-dependent intercellular signaling (Korherr *et al.*, 1997). Meanwhile IL1R2 responsible as an endogenous inhibitor of IL1 signalling (Peters *et al.*, 2013).

Several studies have reported the effects of IL1R1: (i) the polymorphisms in *IL1R1* gene in Chinese Han population could resulted in either reducing (rs956730) or increasing (rs3917225) the risk of head and neck cancer (HNC) (Tian *et al.*,

2020); (ii) global knockdown of IL1R1 lesser the psychosocial stress effects (reduced social interaction and impared working memory), as well as monocyte trafficking to brain (stress lead to inflammatory reaction within central nervous systems); while the restoration of IL1R1 on glutamatergic neurons leads to reoccurance of impairments (DiSabato *et al*., 2020); (iii) deletion of IL1R1 specifically in blood-brain barrier (BBB) ECs lead to reduction in autoimmune encephalomyelitis (EAE) disease severity (EAE is animal model for experimenting inflammatory disease of multiple sclerosis) (Hauptmann *et al.*, 2020); while (iv) increase expression of IL1R1 in tumours of breast cancer patients resemble the failure of anti-estrogen (AE) therapy; while introduction of IL1R1 inhibitors did reduce the activity of cancer stem cells (CSCs) activity during the AE treatment (Sarmiento-Castro *et al.*, 2020). Hence, these studies showed that the expression of IL1R1 is positively related to inflammation level: the increase of IL1R1 expression would portray the high level in the inflammation condition, while the decrease of IL1R1 expression would portray the low level in the inflammation condition.

2.1.3(c) Nuclear factor kappa B subunit 1 (NFκB1)

Sen and Baltimore were first discovered nuclear factor kappa B (NFκB) as a factor that bound to the κ light chain immunoglobulin enhancer in the nuclei of B lymphoid lineage (Sen and Baltimore, 1986a). Later, this characterised NFκB also found to be in phorbol 12-myristate 13-acetate (PMA)-treated Jurkat and Hela cells, suggesting its inducibility capacity and non-specificity towards particular cell types (Sen and Baltimore, 1986b; Maruyama *et al.*, 2016; Rosskopf *et al.*, 2018). NFκB signalling pathway was then reported to play vital roles in controlling various areas: e.g. stress response (Rashid *et al.*, 2017; Lingappan, 2018), apoptosis (Dondelinger *et al.*, 2015;