PREDICTION AND EXPRESSION OF MICRORNA TARGETING INTERLEUKIN-17A IN CHONDROCYTES AND SYNOVIAL FIBROBLASTS ISOLATED FROM OSTEOARTHRITIC KNEE

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

%	Percentage
^{0}C	Degree Celsius
€	Efficiency
ACAN	Aggrecan
ACT1	Act 1 adaptor
ACTB	Beta actin
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
Ago	Argonaute
AMPK	AMP-activated protein kinase
C/EBPβ	CCAAT/ enhancer binding protein beta
C/EBPδ	CCAAT/enhancer binding protein delta
CCL2	C-C motif chemokine ligand 2
CRP	C-reactive protein
CXCL1	C-X-C motif chemokine ligand 1
DEPC	Diethyl polycarbonate
DGRCR8	Drosha and DiGeorge critical region 8
dH20	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
et al.	*et alii* – 'and others'
FGF	Fibroblast growth factor
FLS	Fibroblast-like synoviocytes
GADPH	Glyceraldehyde 3-phosphate dehydrogenase

HA	Hyaluronic acid
IGF	Insulin-like growth factors
IκBα	Inhibitor of nuclear factor kappa B alpha
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-21	Interleukin-21
iNOS	Inducible nitric oxide synthase
JAK-STAT	Janus kinase/signal transducers and activators of transcription
Kcal	Kilocalorie
KEGG	Kyoto encyclopaedia of genes and genome
LKB	Leukotrienes
MAPK	Mitogen-activated protein kinase
MFE	Minimum free energy
min	Minute
miRNA	MicroRNA
mL	Millilitre
MMP	Matrix metalloproteinases
mol	Mole
NCBI	National centre for biotechnology information

NF-κB	Nuclear factor kappa B
ng	Nanogram
NGF	Nerve growth factor
NO2	Nitric oxide
NSAID	Non steroid anti-inflammatory drug
OA	Osteoarthritis
PBS	Phosphate buffer saline
PGE	Prostaglandins
qPCR	Quantitative polymerase chain reaction
r2	Correlation coefficient
SD	Standard deviation
SF	Synovial fluid
SOX6	SRY-Box transcription factor 6
TGF-β	Transforming growth factor beta
Th	T helper
TKA	Total knee arthroplasty
TNF-α	Tumour necrosis factor alpha
TRAF	Tumour necrosis factor receptor-associated factor
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
xg	Gravity
YLD	Years lived with disability
µL	Microlitre
µM	Micromolar

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RAMALAN DAN PENGEKSPRESIAN MIKRORNA YANG MENYASAR INTERLEUKIN-17A PADA KONDROSIT DAN FIBROBLAS SINOVIAL YANG DIISOLASI DARI LUTUT OSTEOARTRITIS

ABSTRAK

Osteoartritis (OA) adalah penyakit degenerasi sendi yang umum dan sering menyebabkan kesakitan dan kecacatan. Disregulasi mikro-RNA (miRNA) dan proses keradangan memainkan peranan penting dalam patogenesis OA, dengan interleukin-17A (IL-17A) menjadi sitokin pro-inflamasi penting yang terlibat dalam degradasi tulang rawan dan keradangan sinovial. Pemahaman tentang regulasi pengisyaratan IL-17A dalam kondrosit dan fibroblas sinovial adalah sangat penting untuk mengungkapkan mekanisme penyakit yang mendasarinya. Penelitian ini bertujuan untuk meramal dan menentukan pengekspresian miRNA yang menyasarkan IL-17A dalam kondrosit dan fibroblas sinovial yang diisolasi dari sendi lutut OA. Kaedah bioinformatik, termasuk TargetScan, miRWalk, dan miRDB digunakan untuk mengenalpasti potensi miRNA yang menyasar IL-17A. MiRNA dengan afiniti pengikatan ramalan tertinggi dan terendah telah dipilih. Analisis server web DIANA-mirPATH digunakan untuk mengenalpasti jalur berpotensi yang dapat disasar oleh miRNA terpilih. Pengekspresian miRNA ini telah dinilai menggunakan kaedah reaksi berantai polimerase kuantitatif (qPCR) dalam kondrosit dan fibroblas sinovial yang diisolasi daripada pesakit OA. Nilai ΔC_T dihitung dalam penelitian ini. Hsa-mir-1913 menunjukkan afiniti pengikatan ramalan tertinggi untuk mRNA IL-17A, sementara hsa-mir-514a-5p menunjukkan afiniti pengikatan yang lebih rendah. Pengekspresian miRNA ini digunakan sebagai ukuran perbandingan untuk mengesahkan ketepatan

ramalan yang diperoleh daripada kaedah bioinformatik. Ramalan sasaran miRNA yang dinyatakan mengenal pasti laluan berkaitan keradangan yang berbeza yang konsisten dengan kajian terdahulu. Analisis qPCR menunjukkan pengekspresian hsa-mir-1913 yang tinggi dalam sampel kondrosit dan fibroblas sinovial (14,36 ± 0,72; 14,12 ± 0,69). Manakala, hsa-mir-514a-5p (-4,65 ± 0,45; -5,05 ± 0,60) menunjukkan pengekspresian yang rendah dalam kedua-dua sampel. Penting untuk diakui bahawa penelitian ini mewakili penyelidikan awal terhadap miRNA yang berpotensi dalam pengawalaturan IL-17A dalam tulang rawan dan fibroblas sinovial pesakit OA. Penelitian lebih lanjut diperlukan untuk memahami mekanisme spesifik dan kesan fungsian daripada miRNA ini dalam patogenesis osteoartritis. Penelitian ini berjaya meramal dan mengesan pengekspresian miRNA yang menyasarkan IL-17A dalam kondrosit dan fibroblas sinovial yang diisolasi dari sendi lutut OA. Pengenalpastian miRNA yang menyasarkan IL-17A dapat memperlihatkan peranan penting miRNA dalam memodulasi proses keradangan dan degeneratif yang terkait dengan OA dan membuka peluang baru untuk penelitian dan pengembangan terapi yang mensasarkan OA.

PREDICTION AND EXPRESSION OF MICRORNA TARGETING IL-17A IN CHONDROCYTES AND SYNOVIAL FIBROBLASTS ISOLATED FROM OSTEOARTHRITIC KNEE

ABSTRACT

Osteoarthritis (OA) is a prevalent degenerative joint disorder associated with pain and disability. Dysregulation of microRNAs (miRNAs) and inflammation processes play significant roles in OA pathogenesis, with interleukin-17A (IL-17A) as the key pro-inflammatory cytokine implicated in the cartilage degradation and synovial inflammation. Understanding the regulation of IL-17A signalling in chondrocytes and synovial fibroblasts are crucial for unravelling the underlying disease mechanisms. This study aimed to predict and determine the expression level of miRNAs targeting IL-17A in chondrocytes and synovial fibroblasts isolated from osteoarthritic knee joints. Bioinformatics tools, including TargetScan, miRWalk, and miRDB were employed to identify potential miRNAs targeting IL-17A. The miRNA with the highest and the lowest predicted binding affinity were selected. DIANA-mirPATH analysis web server was used to identify potential pathways that could be targeted by the selected miRNAs. The expression levels of these miRNAs were assessed via quantitative polymerase chain reaction (qPCR) in isolated chondrocytes and synovial fibroblasts from OA patients. ΔC_T value was calculated in the study. Hsa-mir-1913 exhibited the highest predicted binding affinity to IL-17A mRNA, while hsa-mir-514a-5p showed lower binding affinity. These miRNAs were used as a comparative measure to validate the accuracy of the results obtained from the bioinformatics tools. Target prediction of the expressed miRNAs identified different inflammation-linked

pathways that are consistent with the previous studies. The qPCR analysis demonstrated that hsa-mir-1913 showed consistent high expression in chondrocytes and synovium fibroblasts samples (14.36 ± 0.72; 14.12 ± 0.69). On the other hand, hsa-mir-514a-5p (-4.65 ± 0.45; -5.05 ± 0.60) exhibited lower expression in both samples. It is essential to acknowledge that this study represents a preliminary investigation into miRNAs that potentially regulate IL-17A in chondrocytes and synovial fibroblasts of osteoarthritis patients. Further research is needed to fully understand the specific mechanisms and functional consequences of these miRNAs in the pathogenesis of osteoarthritis. This study successfully predicted and detected the expression of miRNA targeting IL-17A in chondrocytes and synovial fibroblasts isolated from osteoarthritic knee joints. The identification of miRNA targeting IL-17A may highlight the intricate role of miRNAs in modulating the inflammatory and degenerative process associated with OA and open new avenues for future research and development of targeted therapies for OA.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Osteoarthritis (OA) is the most prevalent form of degenerative joint disease and a leading cause of disability among the elderly worldwide (Jang, Lee & Ju, 2021). It poses a substantial and increasing health burden on patients and society due to its rising prevalence worldwide. In the United States (US), statistics show that an estimated 32.5 million people (14%) were diagnosed with OA annually from 2008 to 2014 (Hochberg *et al.*, 2020). Globally, OA exhibits a statistically significant increase in point prevalence (9.3%) and annual incidence rate (8.2%) from 1990 to 2017 (Safiri *et al.*, 2020). Furthermore, the number of people affected by OA globally increased by 48% from 1990 to 2019, ranking it as the 15th leading cause of years lived with disability (YLDs) in 2019 (Hunter *et al.*, 2020).

The escalating prevalence of OA can be attributed to systemic and local biomechanical factors. OA can be categorized into primary and secondary forms. Primary OA is characterized by articular degeneration without a discernible cause, commonly occurring in the elderly due to wear and tear joint development (Bhargava, 2020). On the other hand, secondary OA is influenced by abnormal stress across the joint resulting from post-traumatic causes or underlying conditions like rheumatoid arthritis. Both primary and secondary OA lead to cartilage breakdown and alterations in the underlying bone, including subchondral bone sclerosis, variable degrees of synovial inflammation, and ligament degeneration (Miao *et al.*, 2021).

The interleukin-17 (IL-17) family of cytokines has emerged as a significant contributor to the pathogenesis of OA (Abdel-Naby *et al.*, 2022). Comprising six

cytokines (IL-17A to F), this family plays a pivotal role in orchestrating inflammatory responses and immune regulation in various diseases, including OA. Among the members of the IL-17 family, IL-17A has garnered considerable attention for its involvement in the development and progression of OA. Studies have demonstrated that IL-17A is capable of inducing OA-like transcriptional changes in human OA-derived chondrocytes and synovial fibroblasts (Mimpen *et al.,* 2021). IL-17A, a potent pro-inflammatory cytokine, is produced by immune cells and acts on chondrocytes and synovial fibroblasts in the joint tissues, exerting its effects on these target cells. Specifically, IL-17A can upregulate the gene or protein expression of selected inflammatory mediators such as interleukin (IL)-6, IL-8, C-X-C motif chemokine ligand 1 (CXCL1), C-C motif chemokine ligand 2 (CCL2), cyclooxygenase-2 (COX2), and inducible nitric oxide synthase (iNOS) (Honorati *et al.,* 2002). Moreover, IL-17A has been shown to impact the extracellular matrix (ECM) by increasing the production of matrix metalloproteinases (MMPs) (Hu *et al.,* 2020) further contributing to the perpetuation of inflammation and tissue damage in OA.

MicroRNAs (miRNAs) are small double-stranded RNA molecules that have emerged as powerful post-transcriptional regulators of gene expression, playing crucial roles in various cellular processes, including inflammation and tissue homeostasis (Hutvágner & Zamore, 2002). They function by binding to complementary sequences in mRNA molecules, leading to either mRNA degradation or the repression of translation. IL-17A has been found involved in the pathophysiology of OA, as evidenced by its association with arthritic gene-sets and transcriptional changes that may be regulated by miRNAs (Mimpen *et al.*, 2021). Therefore, understanding the role of miRNAs in regulating IL-17A expression in chondrocytes and synovial fibroblasts of OA patients is of utmost importance in

unraveling the molecular mechanisms driving OA progression. This study aimed to predict and analyze the expression of miRNA that specifically target and regulate IL-17A in chondrocytes and synovial fibroblasts of OA patients.

1.2 Problem statements

OA represents the most common form of degenerative joint disease and stands as a leading cause of disability among the elderly population worldwide. Despite the extensive research on OA, there remains a critical knowledge gap in understanding the molecular mechanisms governing OA pathogenesis (Tong *et al.*, 2022) which hinders the development of targeted and effective therapeutic interventions. Inflammatory cytokines play a critical role in the occurrence and progression of OA. Proinflammatory (IL-1β, tumour necrosis factor alpha [TNF-α], IL-6, IL-15, IL-17 and IL-18] and anti-inflammatory factors (IL-4, insulin-like growth factor and transforming growth factor beta [TGF-β]) have been identified as important contributors to the pathogenesis of OA (Liu *et al.*, 2022). Among the numerous inflammatory cytokines implicated in OA, IL-17A has emerged as a significant contributor to OA disease progression. The role of IL-17A has been linked to the enhanced expression of catabolic factors that are involved in the destruction of cartilage in OA (Ruiz de Morales *et al.*, 2020). However, the specific regulatory mechanism modulating the IL-17A expression within the chondrocytes and synovial fibroblasts remains unknown.

miRNA have recently emerged as pivotal post-transcriptional regulators of gene expression, known to play vital roles in various cellular processes, including inflammation and tissue homeostasis. Their ability to bind to complementary sequences in mRNA molecules leads to either mRNA degradation or the repression of

translation, thereby fine-tuning gene expression. In the context of OA, alterations in miRNA expression have been implicated in influencing the expression of genes involved in cartilage homeostasis, inflammation, and other key processes relevant to disease development. Previous studies have demonstrated a link between miRNA and the pathogenesis of OA through the regulation of chondrogenic processes (Stanciugelu *et al.,* 2022). An imbalance between catabolic and anabolic factors may be caused by abnormal miRNA expression, leading to cartilage degradation when cartilage homeostasis is disturbed (Mobasheri *et al.,* 2017). Despite these findings, the specific miRNAs that target and regulate IL-17A expression in chondrocytes and synovial fibroblasts within OA joint tissues have not been systematically explored.

1.3 Rationale of study

Given the significance of IL-17A in OA pathogenesis and the emerging role of miRNA in regulating gene expression, this study aimed to address the knowledge gap by predicting and analysing the expression of miRNA that specifically target and regulate IL-17A in chondrocytes and synovial fibroblasts of OA patients. By elucidating the miRNA-IL-17A regulatory network, this research endeavoured to provide critical insights into the molecular mechanisms driving OA progression. Understanding the miRNA that regulates IL-17A expression in chondrocytes and synovial fibroblasts may lead to the discovery of novel therapeutic targets. Targeting these miRNAs could offer potential opportunities for developing more precise and effective therapeutic interventions, aimed at mitigating IL-17A-mediated inflammation, and preserving joint tissue integrity.

1.4 Research objectives

1.4.1 General objective

To predict and analyze the expression of microRNAs (miRNAs) that specifically target and regulate IL-17A expression in chondrocytes and synovial fibroblasts of osteoarthritis (OA) patients.

1.4.2 Specific objectives

a. To predict miRNA targeting IL-17A in OA patient's chondrocytes and synovial fibroblasts using bioinformatics tools.
b. To analyze the potential target gene and pathways regulated by the identified miRNA.
c. To determine the expression levels of predicted miRNA targeting IL-17A in OA patient's chondrocytes and synovial fibroblasts using quantitative polymerase chain reaction (qPCR).

CHAPTER 2

LITERATURE REVIEW

2.1 Osteoarthritis (OA)

OA is a debilitating degenerative joint disease with a multifaceted etiology (Yunus, Nordin & Kamal, 2020). It affects millions of people worldwide and involves the progressive loss and degradation of articular cartilage, thickening of subchondral bone, formation of osteophytes, synovial inflammation, degeneration of knee ligaments and menisci, and hypertrophy of the joint capsule (Chen *et al.*, 2017). The condition predominantly stems from the cumulative effects of wear and tear on the joints resulting in the gradual breakdown of articular cartilage (Sen & Hurley, 2023). The severe joint pain associated with OA significantly impacts the daily lives of affected patients limiting their mobility and overall quality of life (Yunus, Nordin & Kamal, 2020).

OA poses a substantial public health challenge, with profound implications for affected individuals, healthcare systems, and the economy (Hamood *et al.*, 2021). Among the various forms, hip and knee OA contribute significantly to this burden and often necessitating surgical interventions to address joint failure. As the global population ages, the incidence of OA rises. The prevalence of knee OA has displayed an upward trend from 2000 to 2023, exerting strain on healthcare systems worldwide. Notably, Asia has exhibited higher prevalence rates than Europe and North America, while South America reported the lowest prevalence (Cui *et al.*, 2020). Women tend to experience higher rates compared to men especially after the age of 50 (Hamood *et al.*, 2021).

OA arises from the intricate interplay of various local and systemic factors, making its etiology complex and multifactorial. Ageing is a significant risk factor as the joint tissues undergo natural wear and tear over time. Genetic predisposition plays a role as some individuals may be more susceptible to cartilage degeneration and OA development. Gender also influences OA risk, with women experiencing higher prevalence rates, potentially influenced by hormonal and biomechanical factors (Sen & Hurley, 2023). Obesity significantly increases the load on weight-bearing joints, accelerating the degenerative process and contributing to OA progression. Occupational factors, especially those involving repetitive joint stress, may also increase the risk of developing OA in certain professions. Furthermore, joint injuries and trauma can lead to post-traumatic OA, which accelerates joint degeneration.

2.2 Pathophysiology of OA

OA commonly occurs in the knee which is the largest synovial joint in the body. The knee joint is a complex structure comprising osseous structures, cartilage, ligaments, and the synovial membrane, enabling the flexion and extension of the legs (Gupton & Terreberry, 2020) (**Error! Reference source not found.**). Excessive load or impact on the joint can lead to damage to the cartilage and bone.

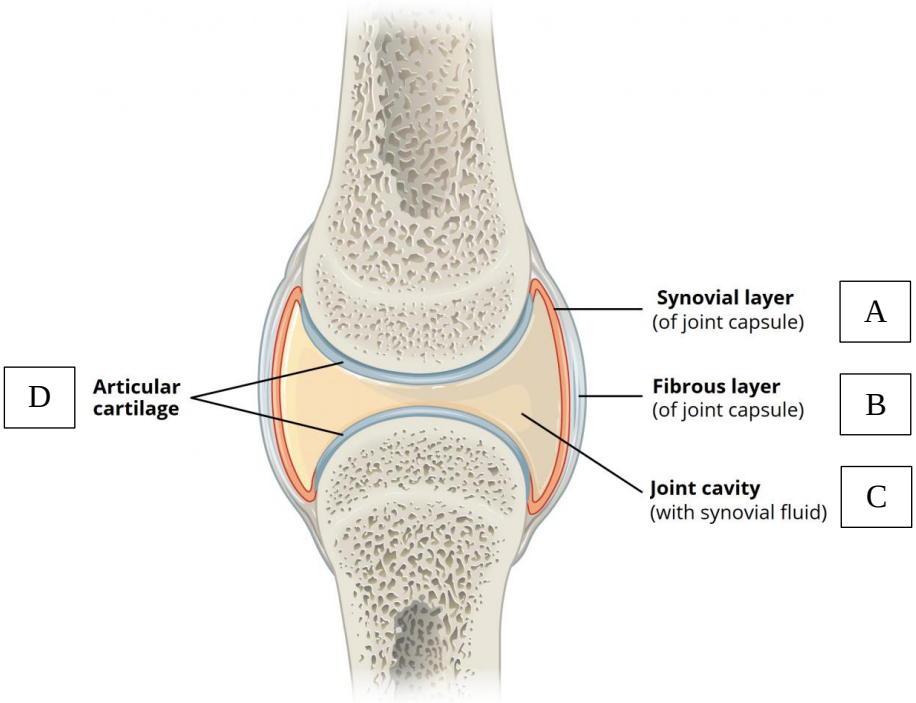

Figure 2.1 General view of knee synovial joints. Adapted from Matt Quinn, (2020) (A) Synovial joint allow for smooth movement between the adjacent bones. (B) The joint is surrounded by a fibrous layer of joint capsule that (C) defines as a joint cavity filled with synovial fluid. (D) The surface of the bones is covered by a thin layer of articular cartilage.

Cartilage, a crucial component of the knee joint contains chondrocytes within an extracellular matrix (ECM) regulated by anabolic influences such as insulin-like growth factors (IGF) I and II, and catabolic influences including interleukin (IL)-1, tumor necrosis factor alpha (TNF-α), and proteinases (Michael, Schlüter-Brust & Eysel, 2010). The ECM is composed of type II collagen and proteoglycans vital for the proliferation and differentiation of damaged joint tissue (Gao *et al.*, 2014). Under normal conditions, the ECM maintains a delicate balance between cartilage degradation and repair processes. Collagens and proteoglycans within the ECM contribute to vascularization and calcification of joint tissue by intertwining with each

other (Eyre, 2002; Poole *et al.*, 2002). However, systemic factors can disrupt this balance leading to overexpression and shift towards irreversible cartilage matrix degradation initiating the early stage of OA.

In the early stage of OA, proliferative chondrocytes form clusters that alter the cellular configuration and composition of the ECM quantitatively. It will further leads to synovitis, inflammation of the joints that as a disease progresses of OA to more advanced stages. The pathophysiological mechanism of OA involves various inflammatory mediators present in the synovial fluid (SF), such as plasma proteins (C-reactive protein [CRP]), prostaglandins (PGE) 2, leukotrienes (LKB) 4, cytokines (TNF, IL [1β, 6, 15, 17, 18, 21]), growth factors (Transforming growth factor beta [TGFβ], fibroblast growth factor [FGF], vascular endothelial growth factor [VEGF], nerve growth factor [NGF]), nitric oxide (NO2), and complement components (Richards *et al.*, 2016; Robinson *et al.*, 2016). These components lead to the secretion of metalloproteinases and other hydrolytic enzymes (Cyclooxygenase [COX] 2 and PGE2), causing progressive cartilage destruction (Sellam & Berenbaum, 2010). As the disease advances, the reparative processes become outweighed by the ongoing cartilage degradation, leading to secondary OA (Primorac *et al.*, 2020; Gupton, Imonugo & Terreberry, 2022). Consequently, the anatomical structure of the knee undergoes pathological changes, including weakening of the osteophyte formation in periarticular muscle, laxity of ligaments, subchondral sclerosis, and synovial effusion (Dulay, Cooper & Dennison, 2015).

2.3 Treatment of OA

In the view of intricate and multifaceted nature of the OA mechanism, there are currently no treatment that can completely cure the disease. As a result, the management of OA has shifted towards interventions focused on decreasing pain, improving joint mobility, and minimizing functional impairment (Richards *et al.,* 2016). This management approach involves pharmacologic, non-pharmacologic treatments, as well as surgical methods targeted prevention and modifying the risk and progression of the disease.

Exercise is a fundamental intervention recommended by clinicians for individuals with OA. Engaging in regular exercise has been demonstrated to have a significant impact, comparable to that of nonsteroidal anti-inflammatory drugs (NSAIDs) in improving joint mobility and providing pain relief with a small to moderate effect (Zhang *et al.,* 2010). However, it is crucial that the exercise regimen is carefully personalized based on the patient's individual tolerance and specific needs. To ensure optimal results and safety, exercise should be implemented under the supervision and guidance of a healthcare professional who can tailor the program to suit the patient's condition and progress.

In the context of pharmacological management, NSAIDs and analgesics like tramadol are commonly used to treat OA providing relief from pain and inflammation. However, their prescription is often limited due to potential adverse effects and risks associated with long-term use. NSAIDs can lead to gastrointestinal complications, ulcers, and bleeding, while also posing concerns for the cardiovascular system, increasing the risk of events like stroke and heart attacks (Ghosh, Alajbegovic & Gomes, 2015). Moreover, long-term NSAID usage may lead to liver and kidney damage. Tramadol, an opioid analgesic, is another option, but it comes with its own

set of potential side effects, including dizziness, nausea, and constipation, and carries a risk of dependency and addiction when used for extended periods (Schug, 2007).

Surgical intervention becomes a consideration when OA reaches a severe stage and conventional treatments found ineffective. Among the alternative surgical options, one approach is intra-articular hyaluronic acid (HA) injection, offering short-term pain relief by replacing reduced HA in the osteoarthritic knee (Ayhan, Kesmezacar & Akgun, 2014). This procedure aids in providing lubrication and enhancing the knee's shock-absorbing properties (Mora, Przkora & Cruz-Almeida, 2018). However, it should be administered cautiously, as frequent injections, more than once every four months can lead to cartilage and joint damage and also elevating the risk of infection (Yu & Hunter, 2015). Another surgical option is total knee arthroplasty (TKA), a common practice for resurfacing the damaged knee joint using prosthetic plastic spacers and metal plates, which are replaced with cartilage (Mohd Noor *et al.*, 2021). While TKA offers a solution for severe cases, its long-term success rate remains inconclusive and may vary based on individual factors (Kirkley *et al.*, 2008; Katz *et al.*, 2013).

2.4 Overview of IL-17

IL-17, initially known as cytotoxic T lymphocyte antigen 8, remained a subject of uncertainty in terms of its functional role for about a decade (Rouvier *et al.*, 1993). However, its significant role came to the forefront with the breakthrough discovery of a novel subset of CD4+ T helper (Th) cells that were found to be associated with the expression of IL-17 (Liu *et al.*, 2013). These cells, termed type 17 T helper cells (Th17), were first identified in 2005 and were recognized as the primary source of IL-17 (Li *et al.*, 2018). This discovery gave rise to extensive research of Th17 cells, aimed at

understanding the development, differentiation, and regulation of this lineage (Amatya, Garg & Gaffen, 2017).

The IL-17 family comprises of six isoforms which were IL17A, IL17B, IL17C, IL17D, IL17E, and IL17F. These isoforms are co-expressed on linked genes and co-produced by Th17. Of these, IL-17A and IL-17E show the highest and lowest homology with other family members (Starnes *et al.*, 2002; Song *et al.*, 2011). Meanwhile, IL17R family consists of five receptor subunits namely IL17RA, IL17RB, IL17RC, IL17RD and IL17RE. The IL-17 signaling cascade initiates with the binding of couple IL-17A/F or combination cytokines to their receptors IL-17RA and IL-17RC. It initiated the activation of Act 1 adaptor (ACT1) protein triggering multiple independent signalling pathways mediated by different tumor necrosis factor receptor-associated factor (TRAF) proteins. TRAF6 activation triggers nuclear factor kappa B (NF-κB), CCAAT/ enhancer binding protein beta (C/EBPβ), CCAAT/enhancer binding protein delta (C/EBPδ), and mitogen-activated protein kinase (MAPK) pathways, resulting in the transcription of inflammatory genes (Amatya, Garg & Gaffen, 2017). In OA, the overexpression of inflammatory genes can lead to the release of pro-inflammatory cytokines and chemokines, promoting the recruitment of immune cells and causing chronic inflammation in the affected joints. This inflammatory response contributes to the destruction of articular cartilage, synovial inflammation, and joint degeneration. Recent evidence has shown detectable IL-17 levels in serum and synovial fluid from OA patients, with a direct correlation to disease severity (Agarwal, Misra & Aggarwal, 2008; Neogi & Zhang, 2013). IL-17 also induces the release of chemokines, contributing to synovial infiltration and cartilage collapse in OA (Honorati *et al.*, 2002).

2.5 Role of IL-17A in OA progression and joint tissue damage

IL-17A, a pro-inflammatory cytokine primarily produced by Th17 cells, plays a pivotal role in the pathogenesis of OA. This cytokine exerts its biological effects by binding to the interleukin-17 receptor (IL-17R) present on various cell types within the joint, including chondrocytes and synovium. Once bound, IL-17A triggers a complex signaling cascade that leads to the release of several inflammatory mediators, such as IL-6, IL-8, CXCL1, CCL2, COX2, iNOS, and MMP (Benderdour *et al.,* 2002; Mimpen *et al.,* 2021). These inflammatory mediators collectively propagate the inflammatory response and contribute to chondrocyte degeneration and synovial tissue degradation, thereby exacerbating OA progression. A recent study reported an increased serum IL-17 level in patients with early knee OA, suggesting its pathogenic role in the disease, with a positive correlation with the severity of knee OA-related pain (Abdel-Naby *et al.,* 2022).

Notably, IL-17A has a direct and detrimental impact on chondrocytes, the essential cells responsible for maintaining cartilage integrity. Upon exposure to IL-17A, chondrocytes undergo activation, leading to the upregulation of MMP. These enzymes induce collagen and aggrecan degradation, resulting in the breakdown of cartilage tissue (Sinkeviciute *et al.,* 2020). Additionally, IL-17A interferes with the synthesis of proteoglycans. Proteoglycans provide hydration and swelling pressure to the cartilage to resist compressive loads. Consequently, the inhibition of proteoglycan synthesis by IL-17A leads to decreased cartilage integrity and compromised mechanical properties of the tissue, further contributing to the progressive breakdown of cartilage in OA (Wojdasiewicz, Poniatowski & Szukiewicz, 2014).

Beyond its effects on chondrocytes, IL-17A also significantly contributes to synovial inflammation, which is a hallmark feature of OA and a key contributor to

joint pain and dysfunction. IL-17A exerts its biological effects by binding to the IL-17 receptor on synovium, which produces pro-inflammatory cytokines (IL-6, and IL-8) and chemokines (CCL20 and CXCL1). These cytokines and chemokines stimulate the recruitment of immune cells (T cells and B cells) that intensify the inflammatory response and perpetuate synovitis (Hattori *et al.,* 2015). The sustained inflammatory environment further damages joint tissues and exacerbates the progression of OA.

2.6 MicroRNAs

MicroRNAs (miRNAs) are a class of short non-coding RNA molecules, typically consisting of about 22 nucleotides in length (O'Brien *et al.,* 2018). They play a pivotal role in the post-transcriptional regulation of gene expression. The prevailing understanding of miRNA function has been focused on their interaction with the 3' untranslated region (UTR) of target messenger RNA (mRNA) molecules, which results in the suppression of protein production from the target mRNA (Ha & Kim, 2014). However, recent research has unveiled a broader regulatory scope of miRNA, demonstrating their ability to interact not only with the 3'-UTR but also with other regions of the target mRNA, such as the 5'-UTR, coding sequences, and gene promoters (Broughton *et al.,* 2016).

The biogenesis of miRNA initiates with the transcription of primary miRNA in the nucleus, where ribonuclease Drosha and DiGeorge Critical Region 8 (DGCR8) processes it into precursor miRNA (Miao *et al.,* 2013). Subsequently, the precursor miRNA is transported to the cytoplasm and undergoes maturation through ribonuclease Dicer. During this process, the passenger strand is degraded, while the mature miRNA strand is retained. The mature miRNA is then loaded onto Argonaute

(Ago) proteins and forms a complex with the GW182 protein, which mediates binding to target mRNA and suppresses their translation (Figure 2.2) (Miao *et al.*, 2013).

miRNAs are versatile molecules with diverse roles in gene regulation. They are known for their well-established functions in controlling gene expression and intriguing capabilities to modulate transcription and translation rates by shuttling between various subcellular compartments (Makarova *et al.*, 2016). miRNA plays a crucial role in altering the expression of specific genes in stimulated immune or bystander cells (Liu & Abraham, 2013), either by targeting multiple genes simultaneously or acting alone on a single gene, leading to both positive and negative regulatory events (Medzhitov & Horng, 2009). During tissue damage or cellular insult, miRNA can trigger cascades of molecular events aimed at promoting tissue repair. Conversely, in response to inflammation, negative feedback pathways are activated to prevent potentially damaging end-stage processes and maintain tissue homeostasis.

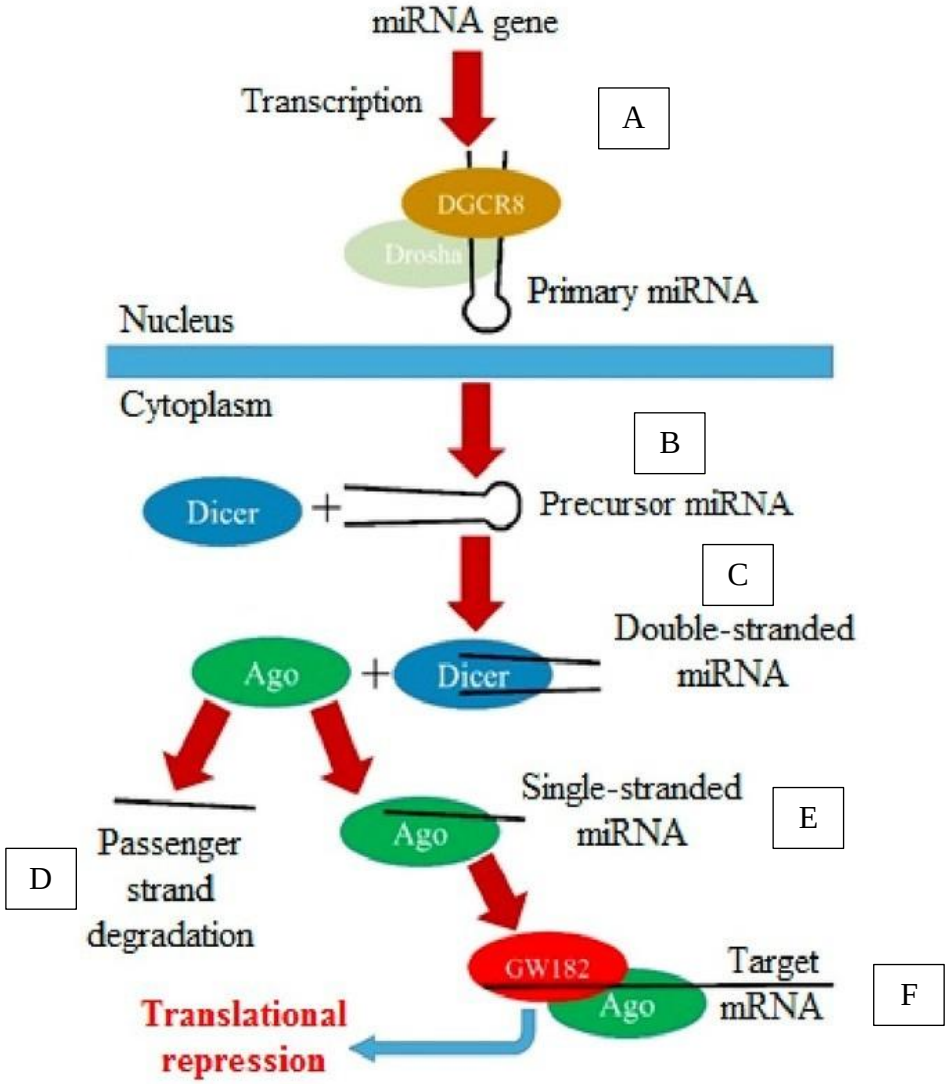

Figure 2.2 The biogenesis of microRNA. Adapted from Panagopoulos & Lambrou, (2018)

(A) miRNA synthesis starts primary miRNA being transcribed in the nucleus from its gene to become precursor miRNA. (B) Precursor miRNA will be transferred into the cytoplasm and processed into (C) mature miRNA. (D) The passenger strand is degraded while (E) the mature strand will be loaded into protein Ago, (F) interact with GW182, bind to its target mRNA, and inhibit its translation.

2.7 Role of inflammatory microRNA in OA

miRNAs play a critical role in precisely regulating various genes involved in cartilage formation and maintenance (Mirzamohammadi, Papaioannou & Kobayashi, 2014). They exert a significant impact on controlling the expression of multiple genes essential for chondrocyte function, extracellular matrix synthesis, and overall cartilage homeostasis. Dysregulation of miRNA expression has been associated with increased production of cartilage matrix-degrading enzymes, such as MMP and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases. For example, the decreased expression of miR-103 in OA cartilage, either directly or indirectly through the inhibition of SOX6, has been linked to the development of OA (Chen & Wu, 2019). Similarly, the downregulation of miR-30a (Ji, Xu, Zhang, *et al.*, 2016) and miR-105 (Ji, Xu, Xu, *et al.*, 2016) have been implicated in excessive production of ADAMTS proteases, disrupting the delicate balance of cartilage matrix turnover and remodelling.

Regarding synovial inflammation, specific miRNAs have been identified as dysregulated in the context of OA. Increased expression of miR-261-5p has been associated with elevated apoptosis, reduced inflammation, and decreased injury in both in vivo and in vitro studies (Jin, Ren & Qi, 2020). Conversely, miR-16 and miR-132 have been reported to be downregulated in the synovial fluid of OA patients compared to controls (Murata *et al.*, 2010). Additionally, the downregulation of miR-33b-3p, miR-140-3p, and miR-671-3p in the serum of OA patients has also been linked to synovitis (Ntoumou *et al.*, 2017). Importantly, miR-381a-3p was found to be upregulated in the synovium of both OA patients and the monosodium iodoacetate (MIA)-injected rat model of OA pain. This upregulation of miR-381a-3p enhanced the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in

cultured human OA fibroblast-like synoviocytes (FLS) by targeting inhibitor of nuclear factor kappa B alpha (IκBα) (Silong Xia, Kang Yan & Yehua Wang, 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview of study

This study aimed to predict and identify miRNA that targets the IL-17A gene in chondrocytes and synovial fibroblasts isolated from OA patients. The flow of the study was presented in Figure 3.1. Initially, miRNA targeting IL-17A mRNA was predicted using in-silico bioinformatics tools. The best and least binding affinity of miRNA were selected for further analysis of their signalling pathways using bioinformatics tools. Next, chondrocytes and synovial fibroblasts were collected from primary OA patients. The collected samples were processed, and the cells were isolated using collagenase or trypsin-EDTA. Subsequently, the cell samples were assessed for cell viability, and total RNA was extracted using Trizol method. The purity of RNA was evaluated using a NanoDrop spectrophotometer, and then the RNA was subjected to cDNA synthesis before being used for quantitative PCR (qPCR) to assess the expression of the predicted miRNA. The data obtained was analysed using SPSS software and reported as mean and standard deviation (SD).

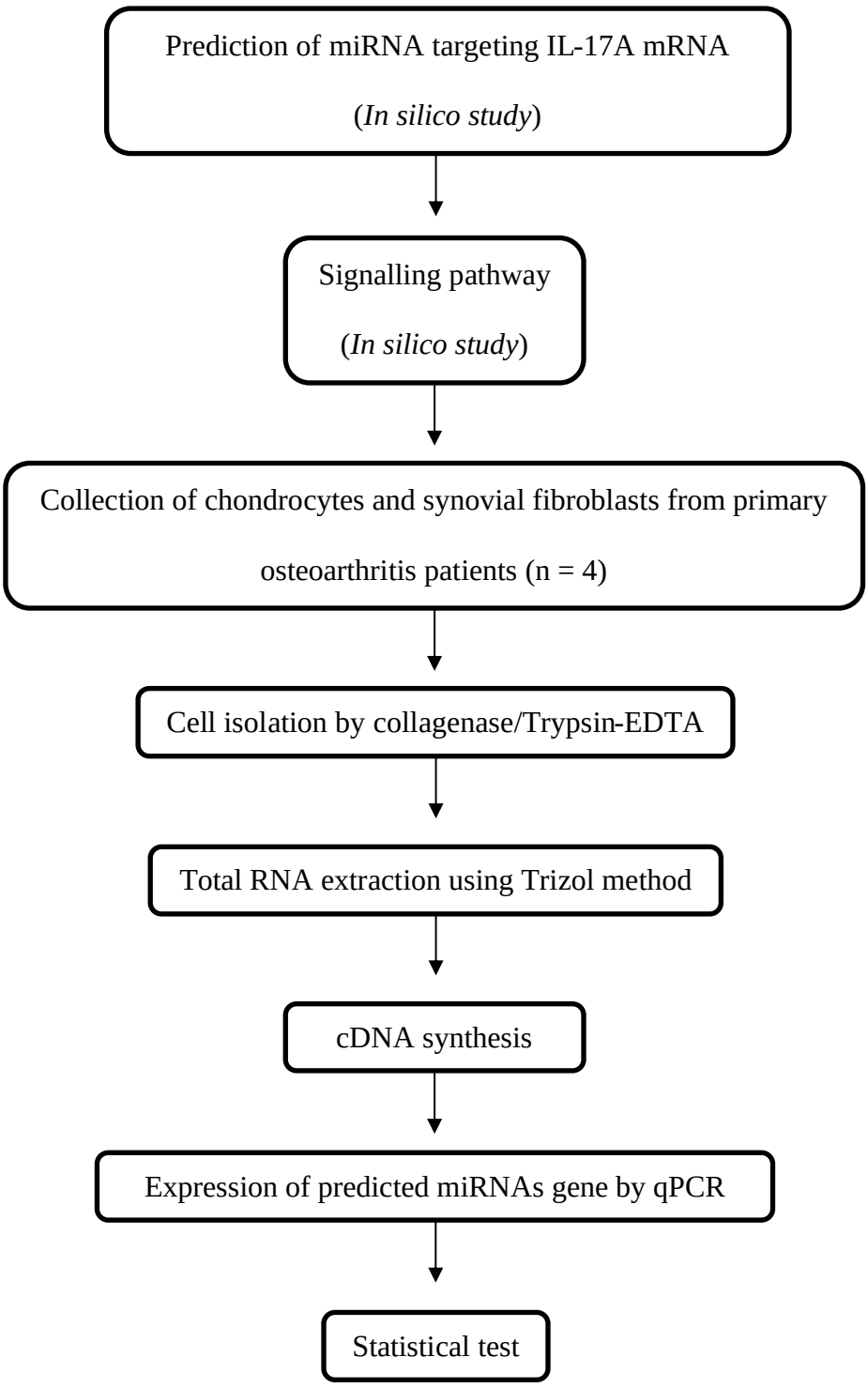

Figure 3.1 The flow chart of the study

3.2 List of chemicals, reagents, kits, and consumables

All chemicals and reagents, kits and consumables used in this study are listed in Table **3.1**, Table **3.2**, and Table **3.3** respectively.

Table 3.1 List of chemicals and reagents

Chemicals and reagents	Manufacturer
Amphotericin B Solution	Gibco, USA
Chloroform	Sigma-Aldrich, USA
Collagenase	Sigma-Aldrich, USA
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco, USA
Absolute Ethanol	HmbG, Germany
Fetal Bovine Serum	Gibco, USA
Isopropyl Alcohol	Sigma-Aldrich, USA
Nuclease Free Water	Qiagen, Germany
Penicillin-Streptomycin	Gibco, USA
Phosphate Buffer Saline (PBS)	Invitrogen, USA
PCR Primers	IDT, Malaysia
Trizol	Invitrogen, USA
Trypan Blue Solution, 0.4%	Gibco, USA
Trypsin EDTA, 0.25%	Gibco, USA
UltraPure™ Diethyl Polycarbonate (DEPC) Treated Water	Invitrogen, USA

Table 3.2 List of kits

Kits	Manufacturer
HiScript® III 1st Strand cDNA Synthesis Kit	Vazyme, China
Luna® Universal qPCR Master Mix	NEB, USA

Table 3.3 List of consumables

Consumables	Manufacturer
8-tube Strip qPCR Tubes with Clear Caps	Cellpro, China
Cell Strainers	Sigma-Aldrich, USA
Centrifuge Tube (15 mL, 50 mL)	SPL Life Sciences, Korea
Gloves	IRONskin, Malaysia
Microcentrifuge Tube (1.5 mL)	Eppendorf, USA
Parafilm	Bemis, USA
Pipette Tip (10 µL, 200 µL, 1000 µL)	Labcon, USA
Scalpel Surgical Blade	LabAider, India
Serological Pipette (10 mL)	Thermo Scientific, USA
Syringe (10 mL)	Terumo, Japan
Syringe Filter (0.22 µL)	TPP, Germany

3.3 List of laboratory equipment and apparatus

All laboratory equipment, apparatus and software used in this study are listed in Table **3.4** and Table **3.5**, respectively.

Table 3.4 List of laboratory equipment and apparatus

Laboratory equipment and apparatus	Manufacturer
4-way Flipper Tube Rack	Thermo Scientific, USA
Autoclave Steriliser	Amerex Instruments, USA
Beaker (100 mL)	HARIO, Japan
Biosafety Cabinet (BSC) Level II	ESCO, USA
CO_2 Incubator	ESCO, USA
Countess™ Cell Counting Chamber Slides	Invitrogen, USA
Countess™ Automated Cell Counter	Invitrogen, USA
Forceps	LabAider, India
Glass Reagent Bottle with Cap (500 mL, 1000 mL)	DURAN, Germany
Laboratory Deep Freezer (-80°C)	Ilshin Lab, Korea
Laboratory Refrigerator	Panasonic, Japan
Laminar Fume Hood	ESCO, USA
Microcentrifuge Machine	Hettich, Germany
MX3000P Real Time PCR	Stratagene, USA
MyCycler Thermal cycler	Bio-Rad, USA
Nanodrop Spectrophotometer	Thermo Scientific, USA
Petri Dish	Thermo Scientific, USA
Pipette (1000 μL, 200 μL, 10 μL)	Satorius, Germany

Pipette Controller	Thermo Scientific, USA
Power Supply	Bio-Rad, USA
Scalpel Blade holder	LabAider, India
Veriti® Termal Cycler	Thermo Scientific, USA
Vortex Mixer	Labinco, Inda
Water Bath	Memmert, Germany

Table 3.5 List of software and database

Software and database	Copyright
DIANA-miRPath v.3.0	(Vlachos *et al.*, 2015)
Mendeley v.1.19.8	Elsevier Inc, USA
Microsoft Word and Microsoft Excel (2016)	Microsoft Corp, USA
miRDB	(Chen & Wang, 2020)
miRWalk	(Sticht *et al.*, 2018)
MX Pro Software	Stratagene, USA
National Centre for Biotechnology Information (NCBI)	NIH, USA
SPSS v.24	IBM, USA
TargetScanHuman v.7.2	(Agarwal *et al.*, 2015)
UNAFold	(Markham & Zuker, 2008)
University of California Santa Cruz (UCSC) Genome Browser	(Kuhn, Haussler & James Kent, 2013)