Regulation of mi-RNAs Target Cancer Genes Between Exercise and Non-exercise in Rat Rheumatoid Arthritis Induction: Pilot Study

Vimolmas Tansathitaya[1] (iD), Witchana Sarasin[2],
Tanapati Phakham[2], Vorthon Sawaswong[3],
Prangwalai Chanchaem[3] and Sunchai Payungporn[3]

[1]College of Sports Science and Technology, Mahidol University, Nakhon Pathom, Thailand.
[2]Center of Excellence in Systems Biology, Faculty of Medicine, Chulalongkorn University,
Bangkok, Thailand. [3]Research Unit of Systems Microbiology, Department of Biochemistry, Faculty
of Medicine, Chulalongkorn University, Bangkok, Thailand.

ABSTRACT

INTRODUCTION: Rheumatoid arthritis is associated with various cancers. Many studies have investigated physical exercise interventions as health improvements to ameliorate the risk of cancer during rheumatoid arthritis diagnosis. Recently, microRNAs were used as biomarkers for health assessment and cancer prediction in rheumatoid arthritis patients.

METHODS: The effects of exercise interventions on serum microRNAs were investigated in pristane-induced arthritis (PIA) rat models. Twelve Sprague-Dawley male rats were divided into 4 groups including non-exercise without PIA (N-EX), non-exercise with PIA (N-EX + PIA), exercise without PIA (EX) and exercise with PIA (EX + PIA). Blood samples were collected at the end of the study period to analyze miRNA biomarkers and target cancer gene predictions.

RESULTS: Four significant Rattus norvegicus (rno-microRNAs) may purpose as tumor suppressors were identified as potential target cancer gene candidate expressions within the 4 comparative interventional exercise groups. One rno-microRNA and target cancer gene candidate was up-regulated and 3 rno-microRNAs and their target cancer genes were down-regulated.

CONCLUSIONS: Exercise interventions affected rno-miRNAs regulated target cancer gene candidates ITPR3, SOCS6, ITGA6, and NKX2-1 as biomarkers for cancer prognosis in rheumatoid arthritis diagnosis.

KEYWORDS: MicroRNA, rheumatoid arthritis, cancer, pristane-induced arthritis

Introduction

Rheumatoid arthritis (RA) is classified as an autoimmune inflammatory condition characterized by pain, swelling, and inflammation of the joints, along with stiffness which can reduce function and impair the overall quality of life.[1] Following the diagnosis of RA, it has been shown that cytokines including IL-6 can affect the prognosis of breast cancer along with inflammation and further comorbidities.[1,2]

A total of 138 cases of lung and prostate solid tumors were recorded within 12 months of RA diagnosis.[3] Furthermore, those patients diagnosed with RA experienced cancer of greater severity than was the case for patients who did not have RA, with the exception of gastric cancer, where RA patients actually had less severe cases than otherwise healthy people.[4] RA patients were also found to be more likely to be diagnosed with lung cancer or lymphoma than other members of the population.[5] In contrast, the incidence of colorectal and breast cancers was shown to be lower in RA patients in comparison to the wider population.[5] It was found that RA patients were 34%

more likely to experience regional or distant cancer than was the case for non-RA patients (*P* = .0003).[6] Moreover, the risk of mortality in RA patients from breast or prostate cancer increased 40% to 50% in contrast to non-RA patients.[6]

Earlier research has examined the incidence of RA and cancer through the use of microRNA biomarkers in studies investigating whether exercise can be used effectively to treat disease.[7,8] It has been argued that miRNA biomarkers might be usefully employed for prognostic evaluations of small lung cancer cachexia as well as the metabolism in cancer cachexia and inflammation in the tissue.[7] Within the context of exercise science, it is believed that exercise can serve as a tool to improve health and support rehabilitation.[9] One study applied a novel exercise science epigenetic technique in which small non-coding microRNAs with a length of around 20 nucleotides served as biomarkers for the purpose of evaluating improvements in the health of RA patients.[10] Another research study investigating the effects of exercise in RA patients revealed no significant differences when the expressions of miRNA-155 and

miRNA-16 were compared between pre- and post-exercise groups and the control. That study also found that miRNA-146a differed significantly from the control group.[8]

It is possible for the target gene silencing mechanisms which facilitate mRNA degradation or translation inhibition to be determined by micro-RNAs.[11,12] Meanwhile, the alternatives of the miRNA-126 target EGFL 7 have been linked to cancer due to the promotion of inflammation and angiogenesis in RA patients.[12,13] Other researchers have reported that cancer could be induced by tumor necrosis factor alpha (TNFα) via the stimulation of miRNA-155, while miRNA-155 expression played a role in the regulation of IL-6 in the synovial fluid of RA patients through the stimulation of neutrophils, macrophages, and T-cells which can lead to the formation of osteoclasts as well as chronic inflammatory effects.[14-16]

Exercise may represent a novel means of mitigating the suffering of RA and cancer patients.[17,18] A number of studies have sought to examine the application of exercise as a means of inhibiting tumorigenesis, limiting infections, and building up resistance to tumors.[19] Furthermore, the symptoms of some cancers, especially breast cancer, can be reduced through an exercise routine of moderate intensity conducted daily for up to an hour.[17] More recently, it has been found that exercise can support the effects of numerous miRNAs which play a role in the suppression of tumors.[20] In the case of bladder, ovarian, colorectal, prostate, breast, and gastric cancers, the circulation of alternative c-miRNA-133 underwent expression in solid tumors, and served to suppress those tumors in patients who maintained regular exercise.[21] Other research findings have indicated that the expression of miR-206 was positively affected by exercise and could limit cell growth in breast cancer through the inhibition of the G1/S transition, thereby achieving the suppression of cell proliferation.[22] The expression of miRNA 21, miRNA Let-7a, and miRNA16 was also enhanced by exercise, while the expression of miRNA15b was inhibited. The effects upon miRNAs duly influenced the relationship with pro-apoptotic target genes whereby a reduction in PTEN, PDCD4, RAS mRNA and anti-apoptotic resulted in an increase in Bcl-2 mRNA when tests were conducted in rats.[23] This research involving rat models examined the influence of exercise in early stage RA as a predictor of cancer in the context of miRNA expression associated with target cancer gene candidates.

Methods

Animals

Twelve 11-week-old Sprague-Dawley male rats were housed at Chulalongkorn University Laboratory Animal Center (CULAC). The rats were assigned to 4 cages with 3 animals per cage and injected with 100 μl of pristane-induced arthritis (PIA)[24,25] at the dorsal side of the tail as the control group with PIA and the exercise group with PIA. The environment was maintained under a 12/12 light-dark cycle at 22°C with ad libitum food and water.

Exercise intervention

After 2 weeks of quarantine and another week of treadmill training preparation, the animals were divided into 4 groups (3 rats per group). The first group was assigned as non-exercise without PIA induction (N-EX). The second group was assigned as non-exercise with PIA induction (N-EX + PIA). The third group received high-speed treadmill exercise (20-25 m/min) for 60 minutes without PIA induction (EX), while the fourth group received high-speed treadmill exercise (20-25 m/min) for 60 minutes with PIA induction (EX + PIA). The animals were weighed every 2 days (Figure 1). Animals in the exercise groups were allowed to rest for 15 minutes when they showed signs of fatigue (stay at the end of the treadmill or be unable to run with sound or electrical stimulation). On day 45, all rats were euthanized by inhalation of CO_2.

Sample collection

EDTA-blood samples were collected from the external jugular vein (Figure 1). Plasma was separated by centrifugation (8 minutes), aliquoted and stored at –80°C until assay. Total RNA was extracted using TRIzol Reagent and miRNeasy Mini Kit (Qiagen), and then quantified and qualified by Agilent 2100/2200 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agarose gel electrophoresis. The RNA samples were dried in a GenTegra tube under vacuum.

Small RNA library preparation

The 3'SR adaptor for Illumina was ligated to the small RNA using the 3'ligation enzyme. To prevent adaptor-dimer formation, the excess 3'SR adaptor was a hybrid with the SR RT primer. The 5'SR adaptor for Illumina was ligated to the small RNA using the 5'ligation enzyme and first-strand cDNA was synthesized using ProtoScript II reverse transcriptase. Each sample was then amplified by PCR using P5 and P7 primers, with both primers carrying sequences that annealed with flow cells to perform bridge PCR and P7 primer carrying a 6-base index allowing for multiplexing. PCR products of ~150 bp were recovered and cleaned using polyacrylamide gel electrophoresis (PAGE). The final DNA library was validated using an Agilent 2100/2200 Bioanalyzer (Agilent Technologies Palo Alto, CA, USA) and quantified by a Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

Small RNA sequencing

Libraries with different indices were multiplexed and loaded on an HiSeq (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2 × 150 paired-end (PE) configuration and image analysis and base calling were conducted by HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) using HiSeq instrument image analysis.
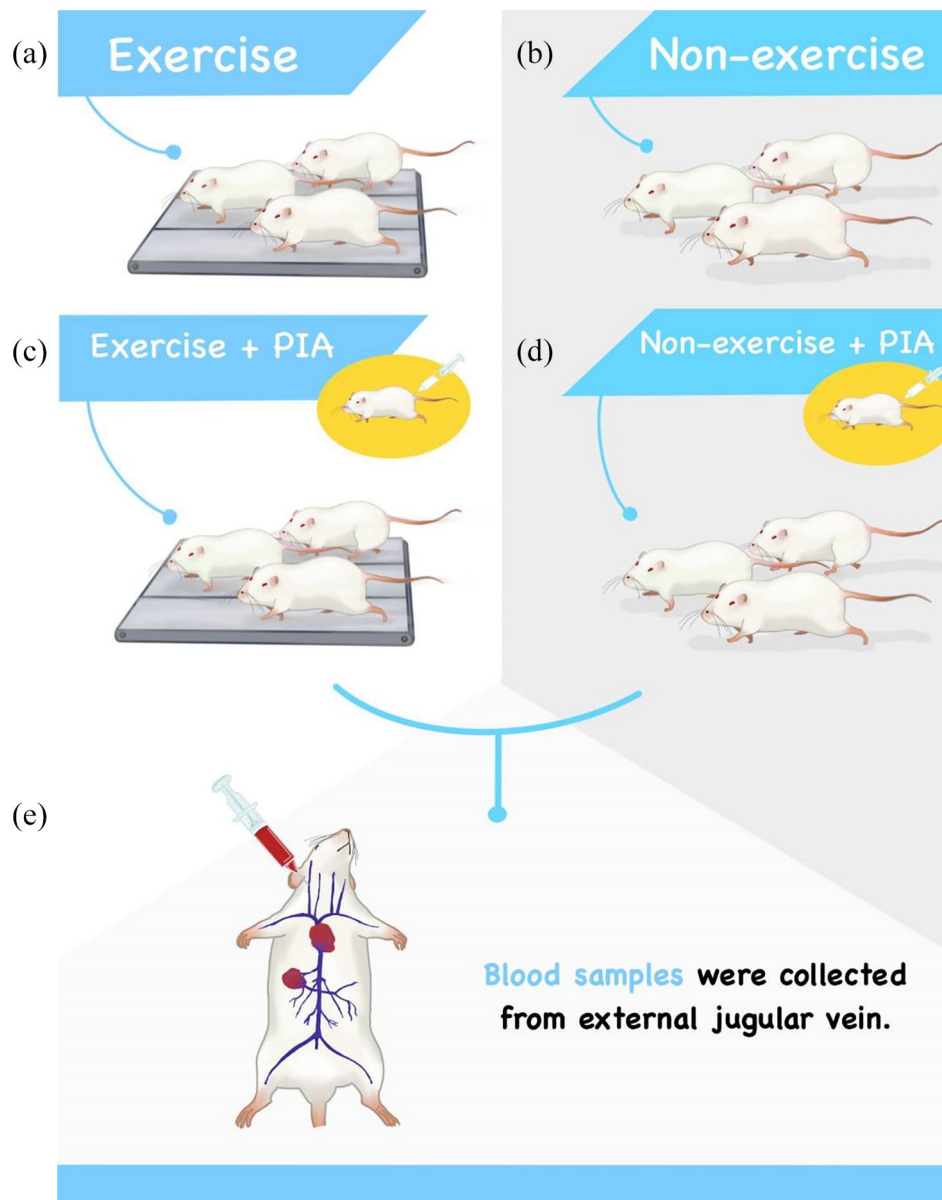
Figure 1. Allocation of the 4 sample groups into 4 exercise conditions: (a) exercise without PIA induction group (EX), (b) exercise with PIA induction group (EX + PIA), (c) non-exercise without PIA induction group (N-EX), (d) non-exercise with PIA induction group (N-EX + PIA), and (e) blood sample collection .

Data analysis

The FASTQ sequences were examined for quality control using FastQC software (v0.10.1). The raw sequencing reads were adaptor trimmed and quality filtered ($<Q30$) by Trimmomatic (v0.30).[26] The cleaned reads were aligned to the reference sequences of *Rattus norvegicus* miRNAs (rno-miRNA) from miRbase (release 22.1) and then annotated.[27] The novel miRNAs were predicted by miRDeep2 software (v2.0.0.8) based on the prediction of secondary structures of microRNA precursors.[28] Differential expression analysis was conducted by DESeq2 (v1.6.3),[29] with differentially expressed microRNAs among groups subsequently selected for further analysis based on *P*-value < .05 and fold change >2. The differentially expressed (DE) microRNA target gene prediction was performed based on

the computational microRNA sequences and the corresponding genomic cDNA sequences using Miranda (v3.3a).[30]

Data interpretation

All rno-miRNAs and target cancer gene results were interpreted using our computerized analysis, with target cancer gene functions reviewed from genecards.org, nature.com, cancerindex.org, ncbi.gov and genome.jp/keg.[31]

Results

Mi-RNA expressions

The volcano plots shown in Figure 2 represent differential expressions of rno-miRNA in a rat model. The 213
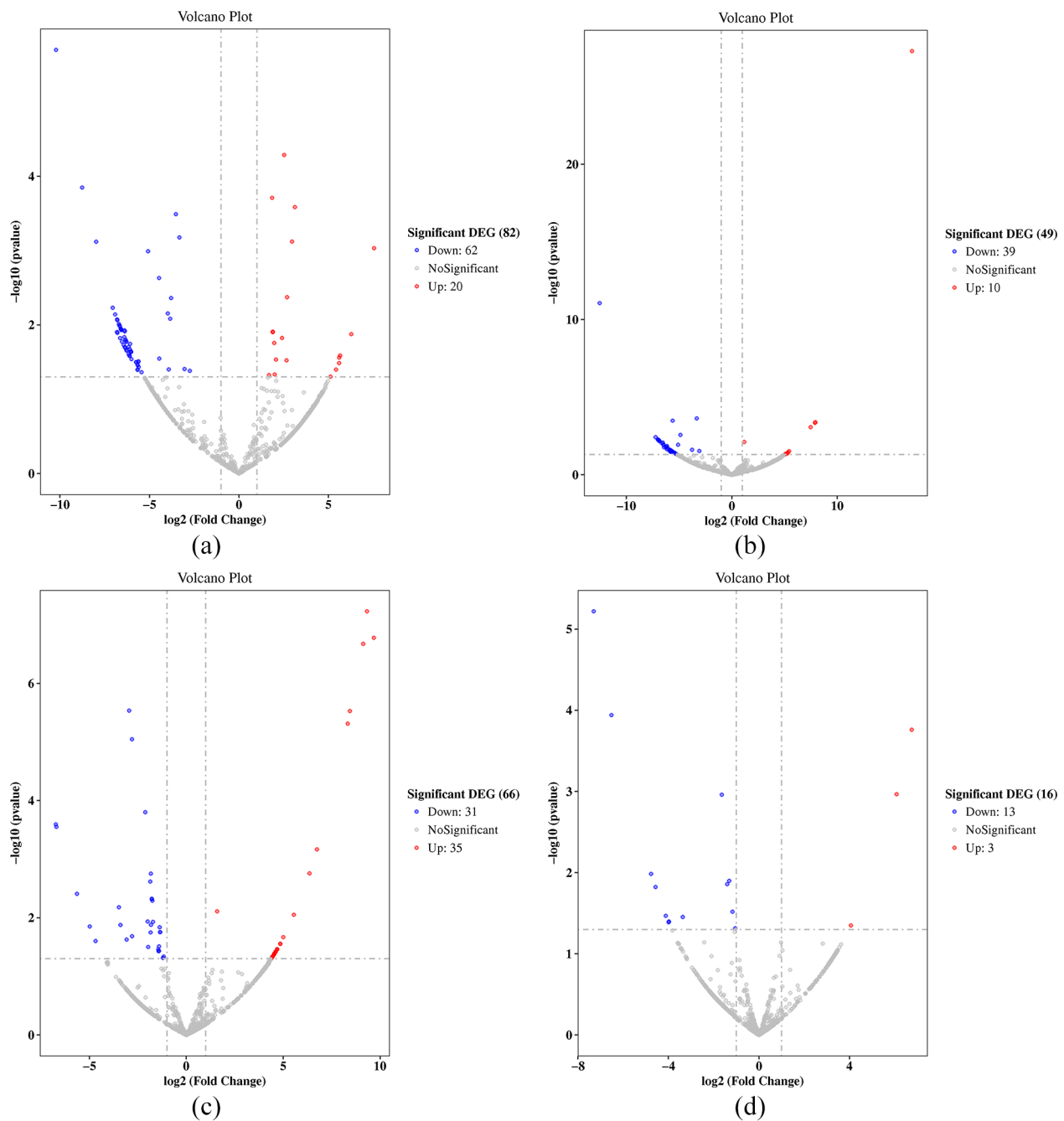
Figure 2. (a) EX versus EX + PIA. A total of 82 rno-miRNAs had significant expressions in the EX group compared to the EX + PIA group. Twenty rno-miRNAs showed up-regulation (red) log2 and 64 rno-miRNAs showed down-regulation (blue) –log2. (b) N-EX + PIA versus EX + PIA. A total of 46 rno-miRNAs had significant expression in the N-EX + PIA group compared to the EX + PIA group. Ten rno-miRNAs showed up-regulation (red) log 2 and 39 rno-miRNAs showed down-regulation (blue) –log2. (c) N-EX versus EX. A total of 66 rno-miRNAs had significant expression in the N-EX group compared to the EX group. Thirty-five rno-miRNAs showed up-regulation (red) log 2 and 31 rno-miRNAs showed down-regulation (blue) –log2. (d) N-EX versus N-EX + PIA. A total of 16 rno-miRNAs had significant expression in the N-EX group compared to the N-EX + PIA group. Three rno-miRNAs showed up-regulation (red) log2 and 13 rno-miRNAs showed down-regulation (blue) –log2.

rno-miRNAs were significant, with 51 known rno-miRNAs and 162 novel rno-RNAs. The 67 rno-miRNAs showed up-regulation, while 145 rno-miRNAs showed down-regulation. Twenty significant rno-miRNAs were up-regulated (log2) and 31 rno-miRNAs were down-regulated (–log2). All known rno-miRNAs are presented in Table 1.

The 4 rno-miRNAs target cancer gene candidates in the comparative interventional exercise groups are listed in Table 2, while the same 4 specific rno-miRNAs with varied other target genes from our computational predictions are shown in Figure 3.

The computation results examined all significant rno-miRNAs from each comparative interventional exercise group and selected significant rno-miRNAs from each comparative group that had only the cancer gene target candidates. We identified gene functions by looking at single gene

Table 1. Comparative exercise intervention groups for known rno-miRNA sequencing: Exercise versus Exercise + PIA.

Table 1a. Exercise versus Exercise + PIA.

RNO-MIRNA	LOG2FOLDCHANGE	*P*-VALUE	EXPRESSION	MATURE_SEQ
rno-miR-877	7.551571032	.000928	Up	uagcuuaucagacugauguugac
rno-miR-466c-5p	5.597666704	.032653	Up	ugugaugugguguauguacaugu
rno-miR-485-3p	5.117861073	.049682	Up	agucauacacggcucuccucucu
rno-miR-128-1-3p	3.128285218	.00026	Up	agucauacacggcucuccucucu
rno-miR-128-2-3p	2.968601278	.000756	Up	ucacagugaaccggucucuuu
rno-miR-340-5p	2.690320347	.004231	Up	uuauaaagcaaugagacugauu
rno-miR-181c-5p	2.658276371	.029973	Up	aacauucaaccugucggugaguu
rno-miR-26a-5p	2.527100973	5.17E−05	Up	uucaaguaauccaggauaggcu
rno-miR-326-3p	2.409908204	.01499	Up	ccucugggcccuuccuccagu
rno-miR-191a-5p	2.069857654	.02928	Up	caacggaaucccaaaagcagcu
rno-miR-676	−2.743780456	.04142	Down	ccguccugagcuugucgagcu
rno-miR-133b-3p	−3.040617641	.039181	Down	uugguccccuucaaccagcuac
rno-miR-192-5p	−3.325176002	.000665	Down	cugaccaugaauugacagcca
rno-miR-122-5p	−3.522260064	.000324	Down	uggagugugacaaugguguuu
rno-miR-149-5p	−3.787945744	.004349	Down	ucuggcuccgugucuucacucc
rno-miR-133a-3p	−3.8398847	.008254	Down	uugguccccuucaaccagcugu
rno-miR-3590-3p	−3.921265018	.03972	Down	uagcacaaugugaaaagagcucc
rno-miR-1b	−3.974299819	.007001	Down	uggaauguaaagaaguauguau
rno-miR-208a-5p	−4.449967622	.028365	Down	gagcuuuuggcccggguuau
rno-miR-1-3p	−4.461695074	.002338	Down	uggaauguaaagaaguguguau
rno-miR-206-3p	−5.076307916	.001021	Down	uggaauguaaggaagugugugg
rno-miR-3585-5p	−6.288747329	.016839	Down	uucacaagaaggugucuuucau
rno-miR-3585-3p	−6.288747329	.016839	Down	ugaacggcccuuguugugaggag
rno-miR-99b-5p	−6.640677728	.015017	Down	cacccguagaaccgaccuugc
rno-miR-103-3p	−6.789162814	.012784	Down	agcagcauuguacagggcuauga
rno-miR-24-3p	−6.813012262	.012453	Down	uggcucaguucagcaggaac

The table shows the regulation of 26 rno-miRNAs in the Exercise group compared to the Exercise + PIA group as 10 up-regulation (log2) and 16 down-regulation (−log 2).

Table 1b. Non-exercise + PIA versus Exercise + PIA.

RNO-MIRNA	LOG2FOLDCHANGE	*P*-VALUE	EXPRESSION	MATURE_SEQ
rno-miR-142-5p	17.08842694	5.18E−28	Up	cccauaaaguagaaagcacuac
rno-miR-877	7.478219813	.000854	Up	guagaggagauggcgcaggggaca
rno-miR-483-5p	5.320541635	.035755	Up	aagacgggagaagagaagggagu
rno-miR-485-3p	5.06650032	.047716	Up	agucauacacggcucuccucucu
rno-miR-99b-5p	−12.52548427	8.76E−12	Down	cacccguagaaccgaccuugc
rno-miR-466b-4-3p	−5.765094629	.032893	Down	uacauacacacauacacacaga

(Continued)

Table 1b. (Continued)

RNO-MIRNA	LOG2FOLDCHANGE	*P*-VALUE	EXPRESSION	MATURE_SEQ
rno-miR-208a-3p	−5.606008068	.000326	Down	auaagacgagcaaaaagcuugu
rno-miR-802-5p	−5.089985923	.011572	Down	ucaguaacaaagauucauccuug
rno-miR-206-3p	−4.869906223	.002686	Down	uggaauguaaggaagugugugg
rno-miR-133a-3p	−3.761392457	.024437	Down	uugguccccuucaaccagcugu
rno-miR-192-5p	−3.322215323	.000233	Down	cugaccuaugaauugacagcca
rno-miR-149-5p	−3.077544148	.029243	Down	ucuggcuccgugucuucacucc

The table shows the regulation of 12 rno-miRNAs in the Non-exercise + PIA group compared to the Exercise + PIA group as 4 up-regulation (log2) and 8 down-regulation (−log 2).

Table 1c: Non-exercise versus Exercise.

RNO-MIRNA	LOG2FOLDCHANGE	*P*-VALUE	EXPRESSION	MATURE_SEQ
rno-miR-208a-5p	9.323598187	5.89E−08	Up	gagcuuuuggcccggguuau
rno-miR-1306-3p	8.329563847	4.87E−06	Up	acguuggcucuggguggugaugg
rno-miR-483-5p	4.608048566	.037996	Up	aagacgggagaagagaagggag
rno-miR-378a-3p	1.591027209	.007763	Up	acuggacuuggagucagaagg
rno-miR-802-5p	−6.735618037	.000255	Down	ucaguaacaaagauucauccuu
rno-miR-3064-3p	−5.642178699	.003898	Down	uuugccacacugcaacaccuua
rno-miR-128-1-3p	−2.949765349	2.92E−06	Down	ucacagugaaccggucucuuu
rno-miR-128-2-3p	−2.803870257	8.99E−06	Down	ucacagugaaccggucucuuu
rno-miR-191a-5p	−2.798641203	.020638	Down	caacggaaucccaaaagcagcu
rno-miR-26a-5p	−2.116197512	.000158	Down	uucaaguaauccaggauaggcu

The table shows the regulation of 10 rno-miRNAs in the Non-exercise group compared to the Exercise group as 4 up-regulation (log2) and 6 down-regulation (−log 2).

Table 1d: Non-exercise versus Non-exercise + PIA.

RNO-MIRNA	LOG2FOLDCHANGE	*P*-VALUE	EXPRESSION	MATURE_SEQ
rno-miR-466b-5p	6.756983533	.000174	Up	augugugugugguaguguccaugu
rno-miR-7b	4.067205338	.044725	Up	uggaagacuugugauuuuguuguu
rno-miR-3064-3p	−3.36593574	.035185	Down	uuugccacacugcaacaccuua

The table shows the regulation of 3 rno-miRNAs in the Non-exercise group compared to the Non-exercise + PIA group as 2 up-regulation (log2) and 1 down-regulation (−log 2).

target functions from all study group results by following references (1) genome.jp/keg.ncbi.gov, (2) genecards.org, and (3) cancerindex.org.ncbi.gov. Once the cancer gene candidates were identified, we chose those genes and their own target rno-miRNAs as our results.

Table 2 shows that the interventional physical exercise groups reduced the risk of cancer. Group 3 (EX) showed the lowest risk of cancer compared to the other study groups. Group 4 (EX + PIA) showed lower risk of leukemia than Group 2 (N-EX + PIA) and lower risk of lung cancer compared to Group 1 (N-EX).

Discussion

Fifty-one rno-miRNAs were expressed with varied target genes. Twenty rno-miRNAs were up-regulated and 31 were down-regulated. Four significant rno-miRNAs targeted 4 cancer gene candidates in the interventional exercise groups. Interestingly, our results also found that expressions of significant rno-miRNAs were different from the hypothesis in 3 out of the 4 interventional exercise groups.

Our results indicated that rno-miRNA-877 up-regulations in the EX group target ITPR 3 expressions associated with proteoglycans in cancer compared to the EX + PIA group. The

Table 2. Rno-miRNA expressions and target cancer gene candidates.

| MIRNA	TARGET GENES	GENE FUNCTIONS	EXERCISE GROUPS	RNO-MIRNA REGULATION	TYPE OF CANCER	CANCER RISK	REFERENCES
rno-miRNA 877	ITPR 3	Tumor suppressor	EX vs EX + PIA groups	Up	Proteoglycans cancer	↓	genome.jp/kegg ncbi.gov
rno-miRNA 466b-4-3p	SOCS 6	Oncogene	N-EX + PIA vs EX + PIA groups	Down	Leukemia	↑	genecards.org
rno-miRNA 128-2-3p	ITGA9	Oncogene	N- EX vs EX groups	Down	Lung cancer	↑	genecards.org
rno-miRNA 3064-3	NKX2-1	Oncogene	N-EX group vs N-EX + PIA groups	Down	Lung cancer	↑	cancerindex.org ncbi.gov
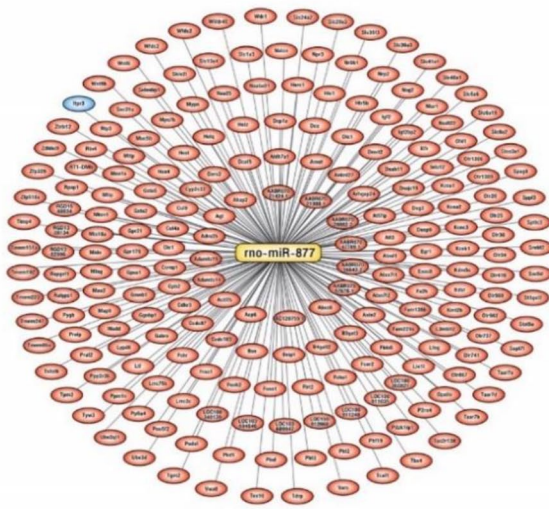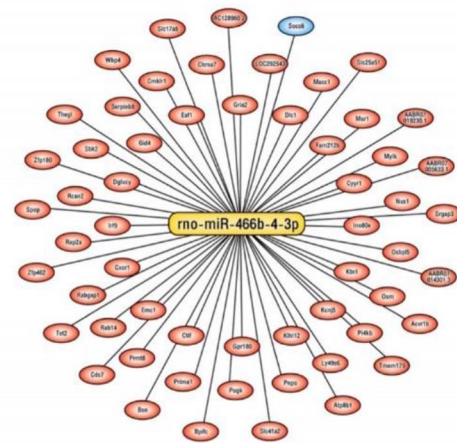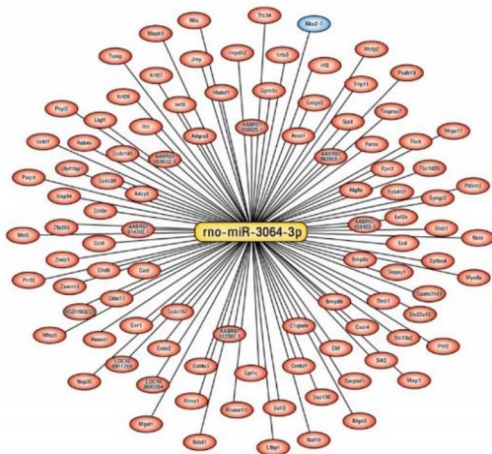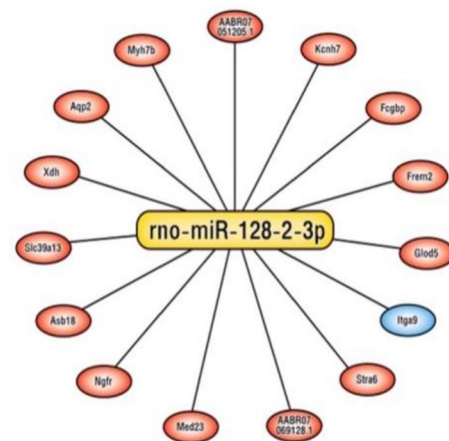
Figure 3. Varied target genes and cancer gene candidates of the 4 rno-miRNAs. (a) EX versus EX + PIA groups. All the target genes of rno-miR-877 (red) and target cancer genes associated with proteoglycans in cancer metastasis (blue). (b) N-EX + PIA versus EX + PIA groups. All the target genes of rno-miR-466b-4-3p (red) and target cancer genes associated with leukemia (blue). (c) N- EX versus EX groups. All the target genes of rno-miR-128-2-3p (red) and target cancer genes associated with lung cancer (blue). (d) N-EX versus N-EX + PIA groups. All the target genes of rno-miR-3064-3p (red) and target cancer genes associated with lung cancer (blue).

up-regulation of rno-miRNA 877 target ITPR 3 in the EX group is associated with proteoglycans in cancer. Results from our computation predictions data and KEGG.jp show that rno-miRNA 877 regulated target specific genes involved in the activation of Teff immune cells and other immune systems.[32] Moreover, rno-miRNA 877 is an activator of CD 28 and also a signal of the IL 2 related proliferator, especially for T-cells.[33] Expression of rno-miRNA 877 and CD 28 also play a role in controlling Treg cell mediation (multi T-cell form) to suppress other antigens including pre-cancer cells from carcinoma and prevent autoimmunity.[34] Without PIA induction, the T-cell and Fas ligand normally control the immune system and tumor necrosis factor (TNF) but with PIA condition, down-regulation of rno-miRNA 877 cannot normally regulate the immune system such as CD 28, T-cells and Fas ligand. Cancer then occurs when cancer genes are expressed after chronic rheumatoid arthritis.[32] Furthermore, some studies suggested that proteoglycans in protein can induce signal transduction via toll-like receptors, interleukins, interferons and chemokines. Exercise may stimulate signaling of proteoglycans to cytokines, and increase the immunity to suppress cancer cells. For example, decorin, as a type of proteoglycans in protein can improve signaling by suppressing cytokine and transforming growth factor (TGF)-ß to impact tumor cell death if exercise occurs.[16]

Rno-miRNA 466b-4-3p that target SOCS 6 expressions associated with leukemia were down-regulated in the N-EX + PIA group compared to the EX + PIA group. Down-regulation in miRNA 466b-4-3p and expression of SOCS 6 related to inside cellular environment change, caused different expression levels in both miRNA and SOCS 6 within the 2 exercise groups. Moreover, the expression of the SOCS family, including SOCS 6, was also altered in rheumatoid arthritis patients and resulted in a change in the cellular status to induce leukemia.[35] Our study did not measure the SOCS 6 expression levels but focused on the possibility of SOCS 6 mechanisms relating to leukemia with exercise. The SOCS 6 regulates the tyrosine kinase receptor associated with immunity, inflammation and leukemia with various mechanisms.[36] Some researchers found that the Flt3 tyrosine kinase receptor was regulated by SOCS 6 mutation that transformed to acute myeloid leukemia (AML), while others suggested that Flt3 regulated SOCS 6.[37] However, both regulation and mechanism results were inconclusive.

Expressions of SOCSs and SOCS 6 associated with rheumatoid arthritis involve several cytokines such as IL-6, IL-10, IL-12 and IL-15 that interfere with Janus kinases (JAKs) signal transducers and activator of transcription (STAT).[35] In chronic rheumatoid arthritis inflammation, SOCSs can alter the expression and regulate JAK/STAT as mutation induces leukemia.[38] By contrast, with exercise JAK/STAT phosphorylation may increase, while satellite cell proliferation differentiated with transcription of the activating cell cycle.[39] Thus, the EX + PIA group could have less SOCS 6 expression than the

N-EX + PIA group. Exercise conditions could induce a healthy cell differentiation cycle, influence the cell microenvironment and suppress the development of leukemia. However, limited information exists about how SOCS 6 alters the expression of leukemia prognosis in rheumatoid arthritis under exercise.

Down-regulation of rno-miRNA 128-2-3p in the N-EX group is associated with target lung cancer cell ITGA 9 expressions (interacts with beta 1 subunit generating α9β1) compared to the EX group. The expression of ITGA 9 occurs in many types of carcinoma cancer tumors.[40] In small cell lung cancer, ITGA 9 can be altered on CpG island by hypermethylation or hypomethylation when lifestyle changes.[40] ITGA 9 is also associated with many types of mechanisms and pathways. Exercise could affect ITGA 9 expression or down-regulation in some miRNAs. Our results in the N-EX group showed greater expression of ITGA 9 than the EX group, possibly related to lung extracellular-matrix cell communications that interacted as critically important in various cell functions.[41] Under exercise, lung functions and cells are changed by body temperature, airway diameter, lung connective tissue, and smooth muscle activation. These factors changed the lung cell environment and may be associated with immunoreactivity in the α 9 subunit in epithelial lung tissue.[42] Our study results indicated that the N-EX group might have more ITGA9 expressions than the EX group but results were unclear. Further investigations are required to confirm these assumptions.

In the final study group, down-regulation of rno-miRNAs 3064-3 in the N-EX group target NKX2-1 expressions compared to the N-EX + PIA group. The NKX2-1 gene encodes homeobox protein or NKX2-1 protein specific to the brain, lungs and thyroid gland, which is involved in the regulation of morphogenesis during early embryonic development.[43] The NKX2-1 gene also encodes the thyroid transcription factor 1 (TTF-1) which explicitly regulates the thyroid gland, lungs, and diencephalon (a part of the brain) as it is a tissue-specific nuclear transcription factor. The protein binds to the thyroglobulin promoter and regulates the expression of thyroid-specific genes. The thyroid gland helps to produce and regulate hormones, particularly hormones involved in brain growth and development. This protein can be used as a marker to determine lung and thyroid gland tumors. The NKX2-1 was induced by activin A via SMAD2 signaling in a human embryonic stem cell differentiation model.[44] Gene mutations and deletions are associated with many genetic diseases, such as benign hereditary chorea, choreoathetosis, congenital hypothyroidism and neonatal respiratory distress (also known as a brain-lung-thyroid syndrome), and probably also associated with thyroid cancer and lung cancer.

NKX2-1, a key molecule in lung development, is highly expressed in non-small cell lung cancer (NSCLC). Two microRNAs (miRNAs) were associated with NKX2-1 as miR-365 which targets NKX2-1 and miR-33a that targets downstream of NKX2-1.[45] Our results showed that the N-EX group

down-regulated rno-miRNA3064-3 compared to the N-EX + PIA group. The rno-miRNA3064-3 targeted the NKX2-1 gene, causing up-regulation of NKX2-1 protein in the N-EX group and enhancing the risk of lung cancer. In N-EX group, NKX2-1 gene induces many gene mechanisms especially on the target genes. Normally, NKX2-1 plays roles in single nucleotide polymorphism (SNP), tumor suppressor or tumor promotors which can bind to varied locus on each gene.[46] NKX2-1can influence some anti-tumorigenic genes to make them become mutation and become lung cancer without lifestyle change.[47] Binding of NKX2-1 to varied gene locus are usually in downstream, proximal promotor (upstream) and intron which also induces lung adenocarcinomas. NKX2-1 can influence EGFR gene become mutation and stimulate lung tumorigenesis. On the other hand, NKX2-1 gene can also suppress KRAS gene to inhibit lung tumorigenesis.[48] In the N-EX group, it could be possible that NKX2-1 gene can play automatically roles to be cancer suppresser to inhibit other carcinoma genes. Thus, expression of NKX2-1 gene is dependent on many factors like gene locus, gene types and base pairs etc.[46]

Furthermore, the N-EX + PIA group was associated with inflammation and body immune system as a possible of PIA body introduction. Stimulation of PIA activated T helper cells (Th17) to regulate the immune system as hemostasis could suppress NKX2-1 gene expression and prevent lung carcinoma.[49] Moreover, PIA injection induced CD$_4$+T cells to play a role as a host immune response to cancer and chronic inflammation.[50] PIA also induced CD$_4$+T cells to develop monocytes/macrophages in response to the pathogens by enhancing CD$_8$+T interaction with B cells.[51]

The CD$_4$+ T cells also modulate immune homeostasis to suppress pro-inflammation and prevent lung cancer formation by suppressing NKX2-1 expressions.[51] However, how the NKX2-1 genes and rno-miRNA 3064-3 relate to exercise requires further investigation.

The strengths of this study were the preliminary integration of candidate target cancer genes and exercise intervention to ameliorate the onset of chronic rheumatoid arthritis. We used our research results in varied rno-miRNAs expressions, together with the possibility of target cancer gene candidates as the mechanism for a prospective study.

Conclusions

Our results indicated that tumor suppresser rno-miRNAs in exercise activity up-regulated target cancer gene expression compared to non-exercise activity in both rheumatoid arthritis and non-rheumatoid arthritis conditions. Due to funding restrictions, our study only investigated the rno-miRNA expressions and used computerized predictions on target cancer gene candidates. Further studies could include (1) measurement of gene expression levels, (2) measurement levels of joint inflammations, and (3) analysis of cancer cell proliferation. Results offer a new modification for epigenetics and exercise science fields to investigate cancer prevention among rheumatoid arthritis patients.

Author Contributions

VT, SP, WS, and TP organized the research ideas. PC was responsible for rno-miRNA isolation, preparation and sequencing. VS, VT, WS, and SP coordinated the data analysis and statistical tests, including writing up the results. Under the writing obligation, VT was also responsible for the manuscript with consultation from SP, VS, TP, WS, and PC. All authors have read and approved the final version of this manuscript.

Ethical Approval

This project was approved by the Chulalongkorn University Animal Care and Use Committee (Approval protocol number 2073010).

ORCID iD

Vimolmas Tansathitaya ⬤ https://orcid.org/0000-0002-7549-8750

REFERENCES

1. Tian G, Liang J-N, Wang Z-Y, Zhou D. Breast cancer risk in rheumatoid arthritis: an update meta-analysis. *Biomed Res Int*. 2014;2014:453012-453019.
2. Drygin D, Ho CB, Omori M, et al. Protein kinase CK2 modulates IL-6 expression in inflammatory breast cancer. *Biochem Biophys Res Commun*. 2011;415:163-167.
3. Askling J, Fored CM, Brandt L, et al. Risks of solid cancers in patients with rheumatoid arthritis and after treatment with tumour necrosis factor antagonists. *Ann Rheum Dis*. 2005;64:1421-1426.
4. Wilton KM, Matteson EL. Malignancy incidence, management, and prevention in patients with rheumatoid arthritis. *Rheumatol Ther*. 2017;4:333-347.
5. Simon TA, Thompson A, Gandhi KK, Hochberg MC, Suissa S. Incidence of malignancy in adult patients with rheumatoid arthritis: a meta-analysis. *Arthritis Res Ther*. 2015;17:212.
6. Nayak P, Luo R, Elting L, Zhao H, Suarez-Almazor ME. Impact of rheumatoid arthritis on the mortality of elderly patients who develop cancer: a population-based study. *Arthritis Care Res*. 2017;69:75-83.
7. Jiang Y, Ghias K, Gupta S, Gupta A. MicroRNAs as potential biomarkers for exercise-based cancer rehabilitation in cancer survivors. *Life*. 2021;11:1439.
8. Özcan ZB, Karaahmetoğlu FS, Çiraci MZ, et al. AB0114 investigation of the effects of exercise on miRNA expressions in patients with rheumatoid arthritis. *Ann Rheum Dis*. 2021;80:1086.1.
9. Uhlemann M, Möbius-Winkler S, Fikenzer S, et al. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. *Eur J Prev Cardiol*. 2014;21:484-491.
10. Boehler JF, Hogarth MW, Barberio MD, et al. Effect of endurance exercise on microRNAs in myositis skeletal muscle - a randomized controlled study. *PLoS One*. 2017;12:e0183292.
11. Macfarlane L-A, Murphy PR. MicroRNA: biogenesis, function and role in cancer. *Curr Genomics*. 2010;11:537-561.
12. Ceribelli A, Yao B, Dominguez-Gutierrez PR, Nahid MA, Satoh M, Chan EK. MicroRNAs in systemic rheumatic diseases. *Arthritis Res Ther*. 2011;13:229.
13. Rajarajeswaran P, Vishnupriya R. Exercise in cancer. *Indian J Med Paediatr Oncol*. 2009;30:61-70.
14. Migita K, Iwanaga N, Izumi Y, et al. TNF-α-induced miR-155 regulates IL-6 signaling in rheumatoid synovial fibroblasts. *BMC Res Notes*. 2017;10:403.
15. Srirangan S, Choy EH. The role of Interleukin 6 in the pathophysiology of rheumatoid arthritis. *Ther Adv Musculoskelet Dis*. 2010;2:247-256.

16. Wang X, Lin Y. Tumor necrosis factor and cancer, buddies or foes?. *Acta Pharmacol Sin*. 2008;29:1275-1288.

17. Li F, Bai M, Xu J, Zhu L, Liu C, Duan R. Long-term exercise alters the profiles of circulating micro-RNAs in the plasma of young women. *Front Physiol*. 2020;11:372.

18. Cooney JK, Law R-J, Matschke V, et al. Benefits of exercise in rheumatoid arthritis. *J Aging Res*. 2011;2011:681640.

19. Silva GJJ, Bye A, El Azzouzi H, Wisløff U. MicroRNAs as important regulators of exercise adaptation. *Prog Cardiovasc Dis*. 2017;60:130-151.

20. Dufresne S, Rébillard A, Muti P, Friedenreich CM, Brenner DR. A review of physical activity and circulating miRNA expression: implications in cancer risk and progression. *Cancer Epidemiol Biomarkers Prev*. 2018;27:11-24.

21. Dong Y, Zhao J, Wu CW, et al. Tumor suppressor functions of miR-133a in colorectal cancer. *Mol Cancer Res*. 2013;11:1051-1060.

22. Pulliero A, You M, Chaluvally-Raghavan P, et al. Anticancer effect of physical activity is mediated by modulation of extracellular microRNA in blood. *Oncotarget*. 2020;11:2106-2119.

23. Liu G, Keeler BE, Zhukareva V, Houlé JD. Cycling exercise affects the expression of apoptosis-associated microRNAs after spinal cord injury in rats. *Exp Neurol*. 2010;226:200-206.

24. Olofsson P, Holmdahl R. Pristane-induced arthritis in the rat. *Methods Mol Med*. 2007;136:255-268.

25. Tuncel J, Haag S, Hoffmann MH, et al. Animal models of rheumatoid arthritis (I): pristane-induced arthritis in the rat. *PLoS One*. 2016;11:e0155936.

26. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30:2114-2120.

27. Kozomara A, Birgaoanu M, Griffiths-Jones S. MiRBase: from microRNA sequences to function. *Nucleic Acids Res*. 2019;47:D155-D162.

28. Friedländer MR, Mackowiak SD, Li N, Chen W, Rajewsky N. MiRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*. 2012;40:37-52.

29. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550.

30. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. *Genome Biology*. 2003;5:R1-R8.

31. Kanehisa M, Araki M, Goto S, et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res*. 2008;36:D480-D484.

32. Colamatteo A, Micillo T, Bruzzaniti S, et al. Metabolism and autoimmune responses: the microRNA connection. *Front Immunol*. 2019;10:1969.

33. van Nieuwenhuijze A, Liston A. Chapter four - the molecular control of regulatory T cell induction. In: Liston, A, ed. *Progress in Molecular Biology and Translational Science*. Vol. 136. Academic Press; 2015;69-97.

34. Moncrieffe H. *Regulatory T Cell* (Tregs). British Society for Immunology. https://www.immunology.org/public-information/bitesized-immunology/cells/regulatory-t-cells-tregs. Accessed on March 10, 2022.

35. Isomäki P, Alanärä T, Isohanni P, et al. The expression of SOCS is altered in rheumatoid arthritis. *Rheumatology*. 2007;46:1538-1546.

36. Kazi JU, Rönnstrand L. FMS-like tyrosine kinase 3/FLT3: from basic science to clinical implications. *Physiol Rev*. 2019;99:1433-1466.

37. Kazi JU, Sun J, Phung B, Zadjali F, Flores-Morales A, Rönnstrand L. Suppressor of cytokine signaling 6 (SOCS6) negatively regulates flt3 signal transduction through direct binding to phosphorylated tyrosines 591 and 919 of flt3. *J Biol Chem*. 2012;287:36509-36517.

38. Keewan E, Matlawska-Wasowska K. The emerging role of suppressors of cytokine signaling (SOCS) in the development and progression of leukemia. *Cancers*. 2021;13:4000.

39. Trenerry MK, Della Gatta PA, Larsen AE, Garnham AP, Cameron-Smith D. Impact of resistance exercise training on interleukin-6 and JAK/STAT in young men. *Muscle Nerve*. 2011;43:385-392.

40. Mostovich LA, Prudnikova TY, Kondratov AG, et al. Integrin alpha9 (ITGA9) expression and epigenetic silencing in human breast tumors. *Cell Adh Migr*. 2011;5:395-401.

41. Basora N, Desloges N, Chang Q, et al. Expression of the α9β1 integrin in human colonic epithelial cells: Resurgence of the fetal phenotype in a subset of colon cancers and adenocarcinoma cell lines. *Int J Cancer*. 1998;75:738-743.

42. Tipton MJ, Kadinopoulos P, de Sa DR, Barwood MJ. Changes in lung function during exercise are independently mediated by increases in deep body temperature. *BMJ Open Sport Exerc Med*. 2017;3:e000210.

43. Pogoriler J, Husain AN. Pulmonary development and pediatric lung diseases. *Pathobiology of Human Disease*. 2014;2575-2587.

44. Phelps CA, Lai S-C, Mu D. Chapter nineteen - roles of thyroid transcription factor 1 in lung cancer biology. In: Litwack, G, ed. *Vitamins and Hormones*. Vol. 106. Academic Press; 2018;517-544.

45. Moisés J, Navarro A, Santasusagna S, et al. NKX2-1 expression as a prognostic marker in early-stage non-small-cell lung cancer. *BMC Pulm Med*. 2017;17:197.

46. Stuart WD, Fink-Baldauf IM, Tomoshige K, Guo M, Maeda Y. CRISPRi-mediated functional analysis of NKX2-1-binding sites in the lung. *Commun Biol*. 2021;4:568.

47. Yamaguchi T, Yanagisawa K, Sugiyama R, et al. NKX2-1/TITF1/TTF-1-Induced ROR1 is required to sustain EGFR survival signaling in lung adenocarcinoma. *Cancer Cell*. 2012;21:348-361.

48. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Investig*. 2015;125:3384-3391.

49. Clement A, Nathan N, Epaud R, Fauroux B, Corvol H. Interstitial lung diseases in children. *Orphanet J Rare Dis*. 2010;5:22.

50. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol*. 2010;28:445-489.

51. Drescher HK, Bartsch LM, Weiskirchen S, Weiskirchen R. Intrahepatic TH17/TReg cells in homeostasis and disease—it's all about the balance. *Front Pharmacol*. 2020;11:588436.