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Integrated Analysis of MicroRNA and mRNA Expression Profiles in Rheumatoid Arthritis Synovial Monocytes

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Objective. **MicroRNAs (miRNAs) play important roles in many biological processes and recent studies have provided growing evidences that miRNA dysregulation might play important roles in the pathogenesis of rheumatoid arthritis (RA). The aim of this study was to investigate the contribution of miRNAs to altered gene expressions in RA.**
Methods. **To investigate whether the differential expression of miRNA in RA could account for the altered expression of certain genes, we compared the different expressions of miRNAs and mRNAs in rheumatoid synovial fluid monocytes with that of normal peripheral blood (PB) monocytes by using a gene expression oligonucleotide microarray and a microRNA microarray.**
Results. **Comparative analysis of the mRNA profiles showed significant different expressions of 430 genes in RA synovial monocytes, of which 303 (70%) were upregulated and 127 (30%) were downregulated, as compared with that of normal PB monocytes. Out of differentially expressed 13 miRNAs, 9 miRNAs were upregulated and 4 miRNAs were downregulated in the RA synovial monocytes. A total of 62 genes were predicted as target genes of the 13 differentially expressed miRNAs in the RA synovial monocytes. Among the 62 miRNA-targeted genes, a few genes such as *GSTM1, VIPR1, PADI4, CDA, IL21R, CCL5, IL7R, STAT4, HTRA1* and *IL18BP* have been reported to be associated with RA.**
Conclusion. **In the present study, we observed that several miRNAs are differentially expressed in RA synovial monocytes, and we suggest that these different expressions of miRNAs may regulate the expression of several genes associated with the pathogenesis of RA.**
Key Words. **MicroRNA, Rheumatoid arthritis, Microarray**

Introduction

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNA molecules of 20-22 nucleotides and they are involved in many biological processes such as development, differentiation, proliferation and apoptosis (1). More than 600 different miRNAs have currently been identified in human, and more than 30% of human genes are regulated by miRNAs. The biological functions of miRNA are mediated by the control of protein production in cells, which is caused by miRNA-induced translational repression or mRNA degradation. miRNAs inhibit mRNA translation or induce mRNA degradation after binding of mature miRNA to miRNA-recognition elements (MREs) within the 3' untranslated region (UTR) of the target genes (2). Several recent reports have demonstrated that miRNAs play important roles in the regulation of the immune response, including the production of inflammatory mediators and the differentiation, proliferation and maturation of immune cells such as lymphocytes (B and T cells) and myeloid cells (monocytes and neutrophils) (2).

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by chronic inflammation of synovial joints, and this is associated with proliferation of synovial cells and infiltration of activated immune cells, including T cells, monocytes and plasma cells, and all this leads to progressive destruction of cartilage and bone. Although the cause of this disease remains unknown, the extensive research done in the past few years on RA has resulted in a dramatic evolution of understanding its pathogenesis. Several inflammatory

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cytokines such as TNF-α, IL-1β, IL-6, IL-12, IL-23 and IL-17A play important roles in the pathogenesis of RA and these cytokines are now established as good therapeutic targets (3).

Previous studies have demonstrated that these inflammatory cytokines regulate the expression of miRNAs, and several miRNAs are also involved in the regulation of cytokine expression in the immune cells. Jing et al. demonstrated that miR-16 binds to AU-rich elements (AREs) in the 3'UTR of unstable mRNA, including TNFα and IL-6, and then this induces TNFα mRNA degradation (4). Also, miR-125b targets the 3'UTR of TNFα transcripts and down-regulates post-transcriptionally the expression of TNFα (5). Taganov et al. have shown an increased expression of miR-146a in the human monocytic THP-1 cells in response to TNFα, IL-1β and lipopolysaccharide (LPS) (6). In that report, miR-146a targets the IL-1 receptor associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), suggesting that increased miRNA-146a might inhibit the effect of IL-1β as a negative feedback mechanism. Further, the abnormal expression of several miRNAs was reported in patients with RA. Both miR-155 and miR-146a were found to be overexpressed in synovial tissues and in the synovial fibroblasts of patients with RA (7,8). Among them, miR-146a was mainly expressed in the CD68+ monocytes of synovial tissues from RA patients. TNFα, IL-1β and LPS induce the expression of miR-155 in RA synovial fibroblasts, and the enforced expression of miR-155 in RA synovial fibroblasts represses the level of matrix metalloproteinase 3 (MMP-3), suggesting that miR-155 may be involved in the modulation of the tissue damage in RA (8). The peripheral blood (PB) mononuclear cells from patients with RA exhibit increased expressions of miR-146a, miR-155, miR-132 and miR-16, as compared with that of healthy controls, and high miR-146a and miR-16 expressions are correlated with active disease in patients with RA, suggesting that the levels of the miR-146a and miR-16 expressions may be a useful marker of disease activity in RA (9). However, the pathogenic roles of miRNAs in RA have been mainly studied in RA synovial fibroblasts, and their roles in monocytes/macrophages have not been well studied.

To examine the abnormal expression of miRNAs in RA synovial monocytes, we compared the different expressions of miRNAs and mRNAs in RA synovial monocytes with that of normal PB monocytes by using a gene expression oligonucleotide microarray and a microRNA microarray. Because synovial monocytes are influenced by inflammatory synovial fluid liberated mainly from synovium of RA, we examined synovial monocytes as cells affected by inflammatory condition of RA. Synovial monocytes are derived mainly from circulating monocytes of PB in RA patients and PB monocytes of RA patients also are exposed to abundant amounts of inflammatory cytokines, therefore, we used PB monocytes of healthy individuals which are exposed to negligible concentrations of inflammatory molecules as a control for synovial monocytes from RA patients. In this study, we found that several miRNAs are differentially expressed in RA synovial monocytes as compared to PB monocytes of healthy individuals, and we suggest that these miRNAs may cause misregulation of important genes involved in the pathogenesis of RA.

Materials and Methods

Cell isolation

PB mononuclear cells were obtained from normal PB of four volunteer donors (two females, two males, 27-48 years old, worked in rheumatology research center of Hanyang University) by performing density gradient centrifugation with Ficoll (Invitrogen, Carlsbad, CA, USA) and using a protocol approved by the Hospital for Rheumatic Disease, Hanyang University Institutional Review Board (IRB). The monocytes were obtained from PB mononuclear cells by using anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA, USA). RA synovial monocytes were obtained from the RA synovial fluid mononuclear cells by using anti-CD14 magnetic beads. Twelve patients were included in this study and all 12 patients fulfilled the revised American College of Rheumatology (ACR) classification criteria for the diagnosis of RA. Their clinical characteristics are summarized in Table 1. This study protocol was approved by IRB of the Hospital for Rheumatic Disease, Hanyang University and we got written informed consents from all participated patients.

mRNA expression profiling

The total RNA was extracted from isolated cells (two healthy donors and six RA patients (RA1-RA6)) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. For quality control, the RNA purity and integrity were evaluated by performing denaturing gel electrophoresis and using the OD 260/280 ratio, and the RNA was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Microarray analysis was performed by Macrogen Inc. (Seoul, Korea) using an Illumina HumanRef-8 v3 Expression BeadChip (Illumina, San Diego, CA, USA). Briefly, the total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated cRNA. Seven hundred and fifty nano gram of labeled cRNA samples were hybri-

Table 1. Clinical and demographic features of the study patients with rheumatoid arthritis

Patients	Sex	Age (years)	Disease duration (years)	ESR (mm/hr)	CRP (mg/l)	Medications
RA1	F	57	1	97	3.72	MTX, PD
RA2	F	53	14	77	5.5	MTX, TAC
RA3	F	33	1	47	2.2	MTX
RA4	F	31	7	No data	No data	PD
RA5	F	67	9	72	1.47	MTX, CYC
RA6	F	31	7	39	0.43	MTX, LEF
RA7	F	69	1	68	1.2	MTX, LEF
RA8	F	65	20	81	1.7	REM
RA9	F	64	2	117	4.6	MTX, SSZ
RA10	M	64	2	69	5.4	MTX, LEF
RA11	F	32	9	52	3.77	SSZ, PD
RA12	F	34	2	87	4.1	MTX, HCQ, SSZ

MTX: methotrexate, PD: prednisolone, TAC: tacrolimus, CYC: cyclosporin, LEF: leflunomide, REM: remicade, SSZ: sulfasalazine, HCQ: hydroxychloroquine

dized to each human-8 expression bead array for 16-18 h at 58°C. The arrays were scanned with an Illumina bead array reader confocal scanner. The array data export processing and analysis were performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8). T-tests and fold changes were applied for searching for significant genes. Statistical significance was adjusted by the Benjamini-Hocbherg multiple-testing correction with the false discovery rate (FDR) at 5%. Hierarchical cluster analysis was performed using complete linkage and the Euclidean distance as a measure of similarity.

MicroRNA expression profiling

We performed miRNA expression profiling in the same set of samples (two healthy donors and six RA patients (RA1-RA6)) that were used in the analysis of mRNA microarray. miRNA microarray analysis was performed by Macrogen Inc. (Seoul, Korea) using the Illumina Human MicroRNA Expression Profiling Assay V.2 (Illumina, San Diego, CA, USA), which contains 1,146 assays for detecting more than 97% of the miRNAs described in the Sanger miRBase database. Briefly, biotinylated cDNAs were prepared from the total RNA using the high-throughput Gene expression profiling DASL Assay (cDNA-mediated annealing, selection, extension and ligation). Fluorescently labeled cDNA PCR products were hybridized to the Illumina Sentrix BeadChip U1536-16 according to the protocols provided by the manufacturer. The arrays were scanned using the Illumina bead array reader confocal scanner. Raw data was extracted using the software provided by the manufacturer (Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8)). T-tests and fold changes were applied for searching for significant genes. Statistical significance was adjusted by the Benjamini-Hocbherg multiple-testing correction with the false discovery rate (FDR) at 5%. Hierarchical cluster analysis was performed using complete linkage and the Euclidean distance as a measure of similarity.

Integrated analysis of the microRNA and mRNA expression profiles

Target genes of the differentially expressed miRNAs were determined using MicroCosm Targets Version 5 (miRBase) from the Sanger institute. To identify the putative miRNA-mRNA target pair, the subsets of miRNAs and mRNAs were characterized by a miRBase predicted regulatory relationship and the negatively correlated expression profiles.

MicroRNA and mRNA quantitative real-time PCR

The total RNA was extracted from isolated cells (four healthy donors and twelve RA patients (RA1-RA12)) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. To validate the results of the microarray analysis, two miRNAs (hsa-miR-34a and has-miR-125a-3p) and four target genes (STAT4, IL7R, VIPR1 and PADI4) were selected for quantitative real-time PCR. Real-time PCR of the target genes was performed in triplicate using the iCycler iQ thermal cycler and detection system (Bio-Rad Laboratories, Hercules, CA, USA) and following the manufacturer's protocols. The expression of the tested gene was normalized relative to the levels of GAPDH. Real-time PCR for miRNA was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay primers for hsa-miR-34a and has-miR-125a-3p (Applied Biosystems,

Foster City, CA, USA). The expression of the tested miRNAs was normalized relative to the levels of the RNU44 controls.

Statistics

Statistically significant differences of the miRNA and mRNA expressions between the two groups were determined with the Mann-Whitney test. p-values less than 0.05 were considered significant.

Results

Differentially expressed microRNAs in the RA synovial monocytes compared to the normal PB monocytes

To examine the difference of the miRNA expression between the RA synovial monocytes and normal PB monocytes, we used the Illumina microRNA expression profiling assay. We identified fourteen differentially expressed miRNAs in the RA synovial monocytes as compared with that of the normal PB monocytes (Fig. 1). A significant difference was defined as a 2-fold change and a p-value <0.05. Among the miRNAs that were differentially expressed in the RA synovial monocytes, HS_194 is a novel miRNA that is not described in the miRBase database (10). We cannot map the possible target genes of this novel HS_194 using miRBase, so this miRNA was excluded from the analysis of miRNA-target gene pairing. Out of thirteen miRNAs, except for HS_194, nine miRNAs were significantly overexpressed more than 2 fold and four miRNAs were underexpressed more than 2 fold in the RA synovial fluid monocytes compared with that of the normal PB monocytes.

Microarray analysis of the gene expression in the RA synovial monocytes and normal PB monocytes

To examine the difference of gene expression profile between RA synovial monocytes and the normal PB monocytes, we performed gene expression profiling using an Illumina Human Ref-8 v3 Expression BeadChip in the same set of samples that were used in the analysis of miRNAs. Comparative analysis of the mRNA profiles showed significantly different expressions (defined as 2-fold change and a p-value <0.05) of 430 genes in the RA synovial monocytes, of which 303 (70%) were upregulated and 127 (30%) were downregulated, as compared with that of the normal PB monocytes. To evaluate the functional meaning of the differentially expressed genes, we analyzed the array data using The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classifications System (http://www.pantherdb. org) (11). The PANTHER classification system classifies genes by their functions, and the PANTHER index is an abbreviated ontology that comprises two types of classifications such as molecular function and biologic process. Among the biological process class, the highly represented genes in the RA synovial monocytes included those involved in cell adhesion, immunity

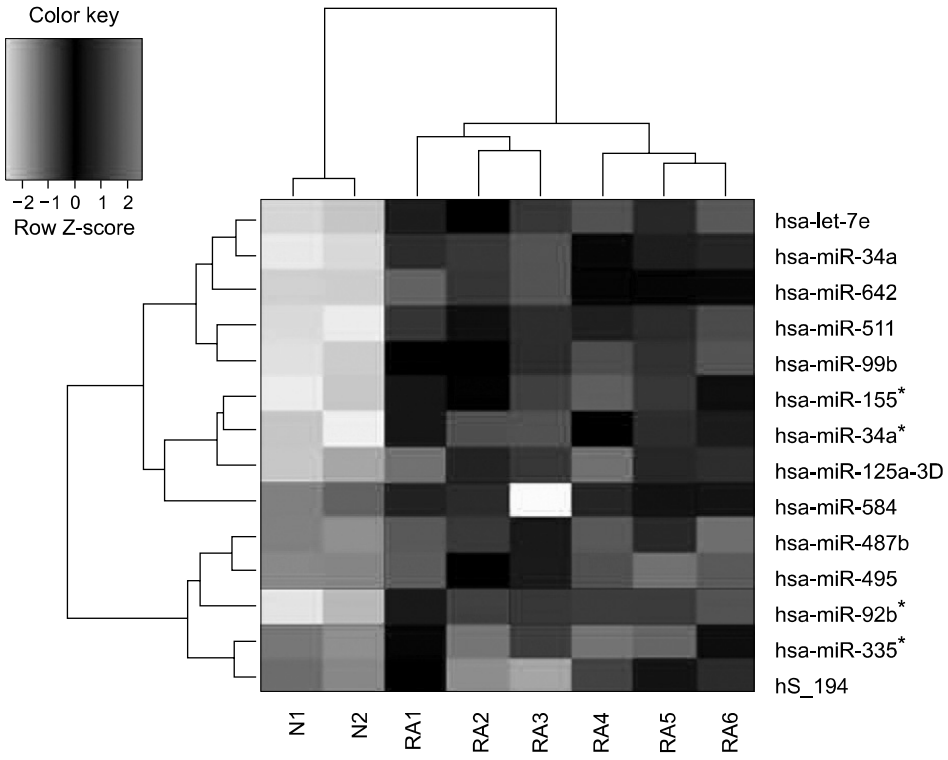

Figure 1. Hierarchical clustering of microRNA expression profile between RA synovial monocytes (RA1-RA6) and normal PB monocytes.

Table 2. Inverse relationship between microRNA and the target mRNA expression identified in the RA synovial monocytes (upregulated microRNAs)

MicroRNA	Gene symbol	Gene name	MicroRNA fold change	mRNA fold change
hsa-let-7e	CD33	CD33 molecule (CD33), transcript variant 1, mRNA.	2.68	-2.37
	TRAF3IP3	TRAF3 interacting protein 3 (TRAF3IP3), mRNA.		-2.93
	PISD	Phosphatidylserine decarboxylase (PISD), mRNA.		-2.06
	CD300LF	CD300 molecule-like family member f (CD300LF), mRNA.		-2.08
hsa-miR-125a-3p	NME3	Non-metastatic cells 3, protein expressed in (NME3), mRNA.	3.81	-2.04
	TRAF3IP3	TRAF3 interacting protein 3 (TRAF3IP3), mRNA.		-2.93
	VIPR1	Vasoactive intestinal peptide receptor 1 (VIPR1), mRNA.		-3.52
	GSTM2	Glutathione S-transferase M2 (muscle) (GSTM2), mRNA.		-6.95
	GSTM1	Glutathione S-transferase M1 (GSTM1), transcript variant 1, mRNA.		-5.13
hsa-miR-155*	IL11RA	Interleukin 11 receptor, alpha (IL11RA), transcript variant 1, mRNA.	8.90	-2.21
	TESC	Tescalcin (TESC), mRNA.		-3.64
	SCPEP1	Serine carboxypeptidase 1 (SCPEP1), mRNA.		-2.13
	TRAF3IP3	TRAF3 interacting protein 3 (TRAF3IP3), mRNA.		-2.93
	CD52	CD52 molecule (CD52), mRNA.		-3.03
hsa-miR-34a	IL11RA	Interleukin 11 receptor, alpha (IL11RA), transcript variant 1, mRNA.	4.11	-2.21
	NME3	Non-metastatic cells 3, protein expressed in (NME3), mRNA.		-2.04
	CDA	Cytidine deaminase (CDA), mRNA.		-2.89
	ZNF467	Zinc finger protein 467 (ZNF467), mRNA.		-2.28
	TRAF3IP3	TRAF3 interacting protein 3 (TRAF3IP3), mRNA.		-2.93
	PADI4	Peptidyl arginine deiminase, type IV (PADI4), mRNA.		-20.95
hsa-miR-34a*	CSTA	Cystatin A (stefin A) (CSTA), mRNA.	7.48	-3.24
	NFE2	Nuclear factor (erythroid-derived 2), 45kDa (NFE2), mRNA.		-3.74
	CD33	CD33 molecule (CD33), transcript variant 1, mRNA.		-2.37
	SCPEP1	Serine carboxypeptidase 1 (SCPEP1), mRNA.		-2.13
	CD300LF	CD300 molecule-like family member f (CD300LF), mRNA.		-2.08
	GSTM2	Glutathione S-transferase M2 (muscle) (GSTM2), mRNA.		-6.95
hsa-miR-511	C14orf159	Chromosome 14 open reading frame 159 (C14orf159), mRNA.	17.72	-2.11
	ALDH1A1	Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1), mRNA.		-2.80
	GSTM2	Glutathione S-transferase M2 (muscle) (GSTM2), mRNA.		-6.95
hsa-miR-642	C6orf192	Chromosome 6 open reading frame 192 (C6orf192), mRNA.	3.79	-2.87
	SLC44A2	Solute carrier family 44, member 2 (SLC44A2), mRNA.		-2.63
	CDH23	Cadherin-like 23 (CDH23), transcript variant 1, mRNA.		-2.13
	PISD	Phosphatidylserine decarboxylase (PISD), mRNA.		-2.06
hsa-miR-92b*	CIDEB	Cell death-inducing DFFA-like effector b (CIDEB), mRNA.	2.03	-3.20
	YPEL3	Yippee-like 3 (Drosophila) (YPEL3), mRNA.		-2.52
	PISD	Phosphatidylserine decarboxylase (PISD), mRNA.		-2.06
	GSTM2	Glutathione S-transferase M2 (muscle) (GSTM2), mRNA.		-6.95
	CD79B	CD79b molecule, immunoglobulin-associated beta (CD79B), transcript variant 3, mRNA.		-2.21
	GSTM1	Glutathione S-transferase M1 (GSTM1), transcript variant 1, mRNA.		-5.13
hsa-miR-99b	CFP	Complement factor properdin (CFP), mRNA.	6.53	-3.59
	ATG16L2	ATG16 autophagy related 16-like 2 (S. cerevisiae) (ATG16L2), mRNA.		-3.33
	IL11RA	Interleukin 11 receptor, alpha (IL11RA), transcript variant 1, mRNA.		-2.21
	MLKL	Mixed lineage kinase domain-like (MLKL), mRNA.		-2.10

and defense, nucleic acid metabolism and signal transduction (Data not shown). Among the molecular function class, the highly represented genes in the RA synovial monocytes included those involved in cell adhesion molecule, defense/immunity protein, receptor, nucleic acid binding and transcription factor (Data not shown).

Identifying the inverse correlation of differentially expressed miRNAs with the expressions of their predicted target genes

MicroiRNAs are generally thought to mediate their biologic functions by mRNA degradation and/or repressing the translation of the mRNA into protein. Recent studies have demon-

Table 3. Inverse relationship between microRNA and the target mRNA expression identified in RA synovial monocytes (downregulated microRNAs)

MicroRNA	Gene symbol	Gene name	MicroRNA fold change	mRNA fold change
hsa-miR-487b	ADFP	Adipose differentiation-related protein (ADFP), mRNA.	-4.71	2.47
	ANKRD9	Ankyrin repeat domain 9 (ANKRD9), mRNA.		3.49
	COLEC12	Collectin sub-family member 12 (COLEC12), mRNA.		7.65
	IDS	Iduronate 2-sulfatase (Hunter syndrome) (IDS), transcript variant 1, mRNA.		2.37
	IL21R	Interleukin 21 receptor (IL21R), transcript variant 2, mRNA.		
	MGAT4A	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A (MGAT4A), mRNA.		2.62 3.68
	NEK6	NIMA (never in mitosis gene a)-related kinase 6 (NEK6), mRNA.		
	PRDX1	Peroxiredoxin 1 (PRDX1), transcript variant 2, mRNA.		2.77
	SMAD6	SMAD family member 6 (SMAD6), mRNA.		2.22
hsa-miR-335*	ANKRD37	Ankyrin repeat domain 37 (ANKRD37), mRNA.		2.62
	ANTXR1	Anthrax toxin receptor 1 (ANTXR1), transcript variant 3, mRNA.	-3.22	2.39
	BCAR3	Breast cancer anti-estrogen resistance 3 (BCAR3), mRNA.		2.38
	CCL5	Chemokine (C-C motif) ligand 5 (CCL5), mRNA.		2.85
	DHRS3	Dehydrogenase/reductase (SDR family) member 3 (DHRS3), mRNA.		6.95
	GPRIN3	GPRIN family member 3 (GPRIN3), mRNA.		8.43
	IL7R	Interleukin 7 receptor (IL7R), mRNA.		2.51
	OLFML2B	Olfactomedin-like 2B (OLFML2B), mRNA.		6.78
	STAT4	Signal transducer and activator of transcription 4 (STAT4), mRNA.		5.26
	ZMYND15	Zinc finger, MYND-type containing 15 (ZMYND15), mRNA.		2.98
hsa-miR-584	ADAMDEC1	ADAM-like, decysin 1 (ADAMDEC1), mRNA.		2.41
	AVPI1	Arginine vasopressin-induced 1 (AVPI1), mRNA.	-3.07	2.85
	BAG3	BCL2-associated athanogene 3 (BAG3), mRNA.		7.52
	MRAS	Muscle RAS oncogene homolog (MRAS), transcript variant 1, mRNA.		2.84
	P4HA2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II (P4HA2), transcript variant 3, mRNA.		2.12 2.29
	PHACTR1	Phosphatase and actin regulator 1 (PHACTR1), mRNA.		
	PRDX1	Peroxiredoxin 1 (PRDX1), transcript variant 2, mRNA.		2.21
	YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide (YWHAH), mRNA.		2.22 3.8
hsa-miR-495	BCAT1	Branched chain aminotransferase 1, cytosolic (BCAT1), mRNA.		
	C17orf58	Chromosome 17 open reading frame 58 (C17orf58), transcript variant 2, mRNA.	-2.34	4.21
	FASN	Fatty acid synthase (FASN), mRNA.		2.01
	HTRA1	HtrA serine peptidase 1 (HTRA1), mRNA.		2.06
	IL18BP	Interleukin 18 binding protein (IL18BP), transcript variant A, mRNA.		2.41
	P4HA2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II (P4HA2), transcript variant 3, mRNA.		3.77 2.29
	PEA15	Phosphoprotein enriched in astrocytes 15 (PEA15), mRNA.		2.6
	TMEM51	Transmembrane protein 51 (TMEM51), mRNA.		11.42
	TUBA1C	Tubulin, alpha 1c (TUBA1C), mRNA.		3.1

strated that the miRNA-induced changes in protein levels are modest and that miRNA induces detectable changes at the mRNA levels in approximately 70% of all regulated proteins (1,12,13). The expression of a given miRNA is inversely correlated with their target gene expressions. We mapped the predicted target genes of the differentially expressed miRNAs (the 13 miRNAs obtained from the microRNA expression profiling assay) to the differentially expressed genes (the 430 genes obtained from gene expression profiling) in the RA synovial monocytes, as compared with that of the normal PB monocytes. The predicted target genes of each of the miRNAs were obtained using MicroCosm Targets Version 5 (miRBase). A total of 62 genes were predicted as the target genes of the 13 differentially expressed miRNAs in the RA synovial monocytes (Table 2, 3). Out of these 62 genes, 34 genes were upregulated and 28 genes were downregulated. These results suggest that only 14% of the differentially expressed genes may be regulated by miRNA-based mechanisms in the RA synovial monocytes.

To further explorer the biologic importance of these differ-

Table 4. Roles of the differentially expressed mRNAs in the RA synovial monocytes

Gene symbol	MicroRNA	Roles in rheumatoid arthritis	Refrences
Decreased			
GSTM1	hsa-miR-125a-3p, hsa-miR-92b*	The presence of a functional allele for GSTM1 associated with a reduced risk of RA	14,15
VIPR1	hsa-miR-125a-3p	Deficient expression in RA patients associated with the predominant proinflammatory Th1 milieu	16,17
PADI4	hsa-miR-34a	Citrullination of selected substrates may end up in dampening of inflammation at the sites of PAD activity	19
		PAD4 contributes to the generation of ACPA specific substrates and is itself a target of autoantibodies in RA.	18
CDA	hsa-miR-34a	Cytidine deaminase levels are raised in serum and synovial fluid in rheumatoid arthritis (RA)	20
Increased			
IL21R	hsa-miR-487b	IL-21R is expressed in RA synovium by RASFs and synovial macrophages	21,22
CCL5	hsa-miR-335*	RANTES is overexpressed in RA SF	23
IL7R	hsa-miR-335*	Enhanced expression of IL-7Ralpha in RA patients contributes significantly to the joint inflammation	24
STAT4	hsa-miR-335*	STAT4, and Jak3 protein expression was generally increased in inflammatory arthritis	25
		A haplotype of STAT4 is associated with increased risk for both rheumatoid arthritis	26,29
HTRA1	hsa-miR-495	HtrA1 contributes to the destruction of extracellular matrix through both direct and indirect mechanisms	27
IL18BP	hsa-miR-495	IL18BP levels were raised in RA	28

entially expressed genes in RA, we search the published reports about the relationship between RA and these genes using PubMed online. Among the 62 miRNA-targeted genes, a few genes such as *GSTM1, VIPR1, PADI4, CDA, IL21R, CCL5, IL7R, STAT4, HTRA1* and *IL18BP* have been reported to be associated with RA (Table 4) (14-29).

Validation of the differentially expressed genes and the miRNAs in RA synovial monocytes by quantitative real-time PCR

To validate the microarray results of the gene expression profile, the expression levels of some genes (*STAT4, IL7R, VIPR1* and *PADI4*) in the isolated monocytes from four healthy donors and twelve RA patients were quantified by performing quantitative real-time PCR. The expression of STAT4 and IL7R were significantly up-regulated in the RA synovial monocytes compared with that of the normal PB monocytes (Fig. 2A). The expression of PADI4 was significantly down-regulated in the RA synovial monocytes and the expression of VIPR1 was down-regulated, but not to a significant degree (Fig. 2A). Consistent with the microarray data of the gene expression profile, similar results were obtained by quantitative real-time PCR.

To verify the microarray results of miRNAs, expressions of hsa-miR-34a and hsa-miR-125a-3p were examined by quantitative real-time PCR. Until now, functions of miRNAs are not extensively studied. For validation of microarray results, hsa-miR-34a and hsa-miR-125a-3p were selected because these two miRNAs were relatively well studied in their biologic functions. The expression of hsa-miR-34a and hsa-miR-125a-3p were significantly up-regulated in the RA synovial monocytes compared with that of the normal PB monocytes (Fig. 2B).

Discussion

In this study, we examined the involvement of differentially expressed miRNAs in the regulation of gene expression in RA synovial monocytes. In parallel to the miRNA expression profiling by the microarray approach, we also proceeded with gene expression profiling by using the microarray approach in the same samples. Parallel examination of the gene expression as well as the miRNA expression in the same set of samples give us insight into the potential interactions between the differently expressed genes and miRNAs in the RA synovial monocytes. Among the 13 differentially regulated miRNAs, 9 miRNAs that include miR-511, miR-155*, miR-34a*, miR-99b, miR-34a, miR-125a-3p, miR-642, let-7e and miR-92b* were upregulated and these corresponded to the decreased expression of 28 target genes in the RA synovial monocytes. Four downregulated miRNAs that included miR-495, miR-584, miR-335* and miR-487b corresponded to the increased expression of 34 target genes in the RA synovial monocytes.

Recent studies have demonstrated dysregulated expression of

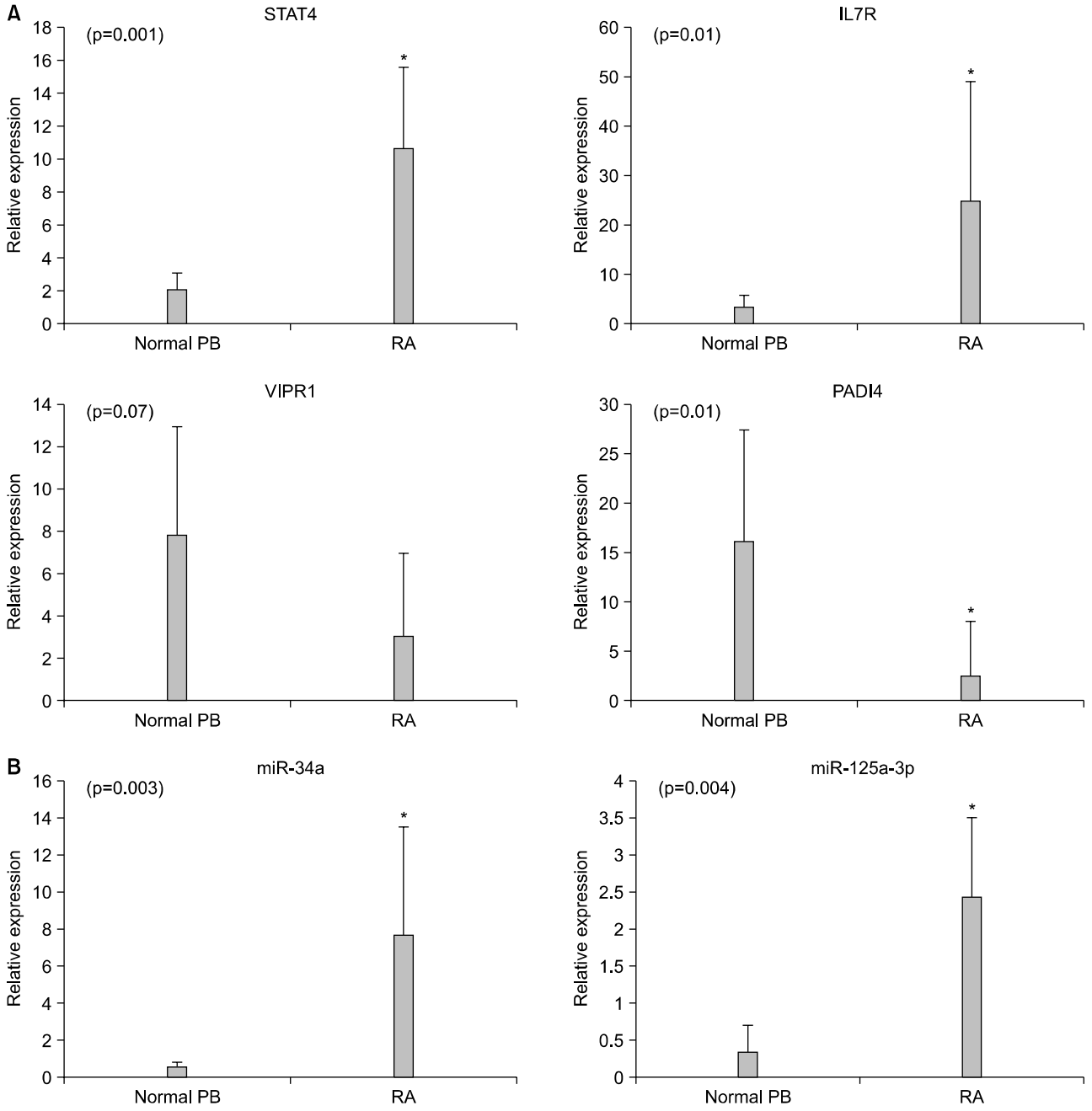

Figure 2. Validation of the differentially expressed genes and miRNAs in the RA synovial monocytes. The array data for mRNAs (A) and miRNAs (B) was validated by performing quantitative real-time PCR on the normal peripheral blood monocytes from four healthy donors and the rheumatoid arthritis synovial fluid monocytes from twelve patients (RA1-RA12). The data is shown as means±SD. *$p < 0.05$.

certain miRNAs such as miR-146a, miR-155, miR-124a, miR-16 and miR-132 in RA patients, suggesting that the abnormal expression profiles of these miRNAs may contribute to the pathogenesis of RA (7-9,30). Most previous studies have examined miRNA expressions in RA synovial tissues, and especially in RA synovial fibroblasts. The expressions of miR-155 and miR-146a in RA synovial tissue were higher than that in osteoarthritis (OA) synovial tissue (7,8). The expression of both miR-155 and miR-146a was higher in the RA synovial fibroblasts than that in OA synovial fibroblasts. TNF-α and IL-1β induced the expression of miR-155, and IL-1β also induced the expression of miR-146a in RA synovial fibroblasts (8). In contrast to the increased expression of both miR-146a and miR-155, miR-124a was underexpressed in the RA synovial fibroblasts compared with that of OA synovial fibroblasts (30). In that report, the expression of monocyte

chemoattractant protein 1 (MCP1) was decreased by induction of miR-124a, suggesting that miR-124a plays a key role in the pathogenesis of RA via regulating the chemokine production of RA synovial fibroblasts. Alsaleh et al. demonstrated that miR-346 functions as a regulator of the inflammatory response in RA via inhibiting the expression of IL-18 (31). In the present study, we identified 13 miRNAs that were either over- or under-expressed in RA synovial monocytes, compared with that of normal PB monocytes. However, these 13 miRNAs are not included in the list of miRNAs reported in the previous studies about dysregulated miRNA expression in RA patients. We believe this discrepancy is most likely due to the organ-specific or cell-type restricted expression of miRNA (32). Unlike our study using RA synovial monocytes, the previous studies for miRNAs in RA have investigated RA synovial fibroblasts or PBMCs. In contrast to the overexpression of miR-155 reported in the previous studies, we observed that miR-155*, which is a 3'-strand counterpart of miR-155, is overexpressed in RA synovial monocytes. miRNA is transcribed as primary precursor transcript (pri-miRNA) that is cleaved into a 60-70 nucleotide precursor hairpin (pre-miRNA) by the nuclear RNase III-like enzyme, termed Drosha. Subsequently, pre-miRNA is further processed by another RNase III-like enzyme, termed Dicer, into a miRNA duplex containing two mature miRNAs (5'- and 3'-strand miRNAs) (33). It was reported that the strand (called miRNA or guide strand) preferentially binds to the miRNA-induced silencing complex (miRISC) and it becomes accumulated and functional, whereas the other one (called miRNA* or passenger strand) is degraded. A recent study suggested that both strands are expressed and accumulated, and the relative expression of the two strands may be tissue dependent (33). Our results indicate the possibility that miR-155*, which is the 3'-strand of miR-155, is preferentially expressed in RA synovial monocytes, as compared with that of miR-155.

Among the list of miRNAs that were differently expressed in RA synovial macrophages, the more interesting miRNAs are miR-34a and miR-125a-3p. In the present study, the predicted target genes of miR-34a are *PADI4* and *CDA*, and expression of both PADI4 and CDA are decreased in RA synovial monocytes compared with that in normal PB monocytes. Unlike our result showing a decreased expression of PADI4 in the RA synovial macrophages, several previous reports have demonstrated an increased expression of PADI4 in the synovial fluid, synovial tissue and PBMCs of RA (34-36). Two reports recently demonstrated that citrullination reduced the pro-inflammatory activity of several chemokines, including CXCL8, CXCL10 and CXCL11, thus suggesting an anti-inflammatory role for peptidyl arginine deiminase (PAD) (19,37). These results suggest that the decreased expression of PAD may be involved in the aggravation of RA activity. Another target gene of miR-34a is cytidine deaminase (CDA), and we observed that the expression of CDA is downregulated in the RA synovial monocytes. In contrast to the downregulation of the CDA expression in our study, previous reports have demonstrated that serum CDA is a marker of inflammatory disease, including RA, and an increased expression of activation-induced cytidine deaminase in the PBMCs from RA patents is positively correlated with the serum levels of anti-CCP and rheumatoid factor (20,38). One possible explanation for the discrepancy between our results and the previous reports is that the medications used by RA patients directly affect the expressions of PADI4 and CDA or the medicines indirectly downregulate the expression of PADI4 and CDA via induction of miR-34a. Our results suggest that miR-34a may work as a natural inhibitor of inflammation in RA. Further experiments are needed to clarify the exact role of miR-34a in RA. VIPR1 is a predicted target gene of miR-125a-3p. Previous reports have demonstrated that vasoactive intestinal peptide (VIP) is the endogenous anti-inflammatory factor and the deficient expression of VIPR1 is observed in the PBMCs from RA patients (16,17). Our results suggest that the increased expression of miR-125a-3p may be one possible mechanism for the deficient VIPR1 expression observed in RA patients.

It is interesting to us that miRNAs belonging to a cluster at 19q13.41, such as miR-99b, let-7e and miR-125a, were overexpressed in the RA synovial monocytes. It has been previously suggested that the combination of chromosomal abnormalities and other types of genetic or epigenetic alterations might contribute to under- and/or over-express miRNAs in malignancies (39). Lionetti et al. demonstrated the over-expression of miR-99b, let-7e and miR-125a in patients with a certain multiple myeloma genetic subtype (TC4) (40). Our observations also suggest the possibility that chromosomal abnormalities may affect the expression of certain miRNAs in RA synovial monocytes, and further study is needed to clarify this possibility.

Also recent study revealed that let-7e repressed Toll-like receptor 4 (TLR4) in macrophages, indicating that the response of macrophages to LPS can be modulated by let-7e (41).

In this study, we predict that several genes related with the pathogenesis of RA, including GSTM1, VIPR1, PADI4, CDA, IL21R, CCL5, IL7R, STAT4, HTRA1 and IL18BP, are targets of the differentially expressed miRNAs in the RA synovial

monocytes, and so we suggest that miR-125a-3p, miR-92b*, miR-34a, miR-487b, miR-335* and miR-495 may play important roles in the pathogenesis of RA. However, integrated analysis of miRNAs and mRNAs using a prediction algorithm such as MicroCosm Targets Version 5 is not yet perfect to investigate the interaction of miRNAs and their target genes due to the inaccuracies in the algorithm of miRNA target prediction and also due to other mechanisms of miRNA such as translational inhibition, which is impossible to detect using a mRNA microarray. Also the low number of samples analysed (2 controls and 6 cases in the microarray studies, and 4 controls and 12 cases in the replication study) is a major limitation in this study.

In the present study, we observed that several miRNAs are differentially expressed in RA synovial monocytes, and we suggest that these altered miRNAs may regulate the expression of several genes associated with the pathogenesis of RA. In this study, we provide a systematic approach for investigating the miRNA-regulated gene expression profile in rheumatic diseases such as RA. In addition to the integrated analysis of miRNAs and mRNA used in the present study, a future study that uses genetic manipulation such as the deletion or over-expression of miRNAs will provide further evidence that these altered miRNAs play important roles in the pathogenesis of RA.

Conclusion

In the present study, we observed that several miRNAs are differentially expressed in RA synovial monocytes, and we suggest that these altered miRNAs may regulate the expression of several genes associated with the pathogenesis of RA. In this study, we provide a systematic approach for investigating the miRNA-regulated gene expression profile in rheumatic diseases such as RA. In addition to the integrated analysis of miRNAs and mRNA used in the present study, a future study that uses genetic manipulation such as the deletion or over-expression of miRNAs will provide further evidence that these altered miRNAs play important roles in the pathogenesis of RA.

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