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Development and Validation of a microRNA Panel to Differentiate between Patients with Rheumatoid Arthritis or Systemic Lupus Erythematosus and Controls

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Abstract

Objective: MicroRNAs (miRNAs) are short non-coding RNAs that regulate genes and are both biomarkers and mediators of disease. We used small RNA (sRNA) sequencing and machine learning methodology to develop a miRNA panel to reliably differentiate between rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) and control subjects.

Methods: Plasma samples from 167 RA and 91 control subjects frequency-matched for age, race and sex were used for sRNA sequencing. TIGER was used to analyze miRNAs. DESeq2 and random forest analyses were used to identify a prioritized list of miRNAs differentially expressed in patients with RA. Prioritized miRNAs were validated by quantitative PCR, and lasso and logistic regression were used to select the final panel of six miRNAs that best differentiated RA from controls. The panel was validated in a separate cohort of 12 SLE, 32 RA and 32 control subjects. Panel efficacy was assessed by area under the receiver operative characteristic curve (AUC) analyses.

Results: The final panel included miR-22-3p, miR-24-3p, miR-96-5p, miR-134-5p, miR-140-3p, and miR-627-5p. The panel differentiated RA from control subjects in discovery (AUC=0.81) and validation cohorts (AUC=0.71), seronegative RA (AUC=0.84), RA remission (AUC=0.85), and SLE patients (AUC=0.80) versus controls. Pathway analysis showed upstream regulators and targets of panel miRNAs are associated with pathways implicated in RA pathogenesis.

Conclusion: A miRNA panel identified by a bioinformatic approach differentiated between RA or SLE patients and control subjects. The panel may represent an autoimmunity signature, perhaps related to inflammatory arthritis, which is not dependent on active disease or seropositivity.

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MicroRNAs are small non-coding RNAs that are important gene regulators and serve as biomarkers of disease. As gene regulators miRNAs can destabilize messenger RNAs (mRNAs) and block translation typically by binding to the 3' untranslated region of the mRNA with a complementary seed region near the miRNA 5' end (1, 2). miRNAs are found within cells, but also circulate in plasma protected from degradation by exosomes (3), microvesicles (4), lipoproproteins (5) and RNA-binding proteins (6). Moreover, miRNAs within these bodies can be transported to recipient cells (5) to regulate genes. Plasma miRNAs are stable in stored samples (7) and are more practical for use as biomarkers than miRNAs in specific cell types due to ease of isolation. Many studies, predominantly in cancer, show that miRNAs can be helpful diagnostic and prognostic biomarkers, particularly when used in a panel composed of multiple miRNAs (8-11).

We and others have found several plasma miRNAs which are differentially altered among patients with rheumatoid arthritis (RA) (12-14). However, most prior studies examined a few miRNAs with known relevant function using polymerase chain reaction (PCR) or used small arrays. Small RNA sequencing provides the ability to evaluate many more miRNAs in an unbiased fashion. Thus, small RNA sequencing could reveal novel miRNA signatures of RA and potentially provide mechanistic insights into disease pathogenesis. Our objective was to determine if a panel of miRNAs derived from small RNA sequencing could differentiate between patients with RA and control subjects, and if that panel validated in a separate RA cohort and was unique to RA or shared with another autoimmune disease: systemic lupus erythematosus (SLE). Additionally, we used pathway analysis to evaluate if these miRNAs have common disease-related upstream regulators which could affect their expression and if the miRNAs could affect RA-related pathways.

MATERIALS AND METHODS

Overview

We used a discovery cohort of RA and control subjects to perform small RNA sequencing for identification of candidate panel miRNAs. Potential candidate miRNAs differentiating RA from controls were prioritized using univariable differential expression analysis (R package 'DESeq2') and a multivariable random forest analysis. These prioritized candidates were validated by quantitative PCR (qPCR). Then, cross-validation lasso with logistic regression was used to further reduce validated miRNA candidates to a small panel of miRNAs which provided best discrimination between RA and control subjects based on area under the receiver operating characteristic curves (AUC). This reduced panel of miRNAs was then externally validated in an independent cohort of patients with RA and control subjects by qPCR. The panel was tested also in a small number of patients with SLE to see if it similarly differentiated between SLE and control subjects.

Study population

The discovery cohort included 167 patients with RA and 91 control subjects frequency-matched for age, race and sex from a prior cross-sectional study (15). The validation cohort included 32 patients with RA and 32 control subjects frequency-matched for age, race and

sex from another prior crosssectional study (16), and twelve patients with SLE from another prior study (17).

Recruitment and study procedures were described previously (15-17), and will be detailed briefly. For the discovery and validation cohorts, subjects were 18 years of age or older. Patients with RA met classification criteria for RA (18), patients with SLE met classification criteria for SLE (19), and control subjects did not have a diagnosis of inflammatory autoimmune disease. Additionally, in the RA validation cohort, which was originally studied to examine the relationship between RA and structural and functional cardiac abnormalities, participants with current or prior heart failure, ischemic cardiovascular disease, structural cardiac disease, atrial fibrillation, estimated glomerular filtration rate <60ml/min, gadolinium hypersensitivity, pregnancy or breast feeding, or inability to have MRI were excluded from the study (16). Studies were approved by the Vanderbilt Institutional Review Board (IRB# 000567, 120314, and 990111) and all subjects gave written informed consent.

Clinical and laboratory Information

We collected clinical information and laboratory measurements as previously described (15, 16). RA disease activity was determined by the 28 joint count disease activity score (DAS28) using erythrocyte sedimentation rate (ESR) (20). High-sensitivity C-reactive protein (CRP) concentrations and ESR were measured by the Vanderbilt University Medical Center Clinical Laboratory.

Small RNA sequencing and microRNA alignments

Total RNA was extracted from stored plasma using Total RNA Purification Kits (Norgen). Libraries were prepared using TruSeq Small RNA Library Preparation Kits (Illumina). RNA extractions and library preparations were performed with both RA and control subject samples in each batch. Libraries were assessed for quality and size selected for approximately 128 to 157 nucleotides in length including adaptors by Pippin Prep (Sage Science) in the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core facility.

The cDNA libraries were sequenced using an Illumina NextSeq500 instrument by the VANTAGE core facility. TIGER (“Tools for Integrative Genome analysis of Extracellular sRNAs”), an in-house small RNA sequencing analysis pipeline (21), was used to analyze sequence data. In brief, high quality reads were demultiplexed using Illumina’s CASAVA 1.8 pipeline and 3’ adapters were trimmed using Cutadapt (22). Reads shorter than 16 nucleotides after adapter trimming were discarded. Three non-templated nucleotide addition (NTA) isoforms of each sRNA read were generated by removing 1, 2 or 3 bases from 3’ terminal. All four isoforms, including three NTA isoforms and original read, were aligned to the human genome (hg19) using Bowtie (23) allowing one mismatch. A sRNA read was identified as a miRNA if its mapped starting position matches any of the first 3 positions from the 5’ end of a miRNA based on the miRNA genome coordinates from miRBase (version 21,http://www.mirbase.org).

MicroRNA analyses

To obtain a prioritized group of miRNAs that were potential candidates for the panel we used both DESeq2 (24), which is Wald test based but can adjust for batch effect and other covariates; and random forest analysis, which is a machine learning method, to capture a prioritized list of candidate miRNAs for the panel. We assumed that many of the miRNAs would overlap, but that using both would help broaden potential candidates.

Sequencing read counts were normalized to total sRNA reads sequenced which passed quality control (reads per total read). Differentially expressed miRNAs were compared by DESeq2 (24), adjusting for age, race, sex, and batch effect. Benjamini-Hochberg adjusted p-values were used to select the 15 most significantly differentially expressed miRNAs (based on P value) for qPCR validation and further model development.

Random forest analyses, which allows for nonlinear relationship between disease status and miRNAs, were conducted to select miRNAs that are most important in separating RA and control subjects. The cross-validated prediction performance of models was evaluated with sequentially reduced number of predictors (ranked by variable importance) via a nested cross-validation procedure. Based on importance score, the top 15 miRNAs were selected for qPCR validation and further model development.

Using qPCR-based plasma concentrations of the miRNAs, we used lasso regularization with logistic regression to select a parsimonious final miRNA panel that maximized discrimination between RA and controls. This panel of miRNAs was validated using qPCR in a separate cohort of 12 patients with SLE, 32 patients with RA and 32 control subjects. Panel efficacy was assessed by area under the receiver operating characteristic curve (AUC) analyses.

qPCR validation

The same plasma samples used for small RNA sequencing were also used for qPCR validation. A cocktail of three *C. elegans* miRNA mimics (cel-miR-39, cel-miR-54, and cel-miR238; Qiagen) was added after the initial lysis step as a spike-in standard for normalizing RNA extraction efficiency. cDNA was prepared using qScript microRNA cDNA synthesis kits (Quantabio). Individual PCR assays (Quanta), and PerfeCTa SYBR green supermix for iQ (Quantabio) were used for qPCR. Plasma miRNA concentrations were determined from standard dilution curves of a DNA mimic and normalized to the spike-in standard. Samples were excluded from analysis if the Ct values of the spike-in standard exceeded one Ct from the median.

General statistical methods

Descriptive statistics were calculated as median [interquartile range] for continuous variables, and frequency and proportions for categorical variables. Wilcoxon’s rank sum tests were used to compare continuous variables and Pearson’s chi-square test to compare categorical variables. PCR-based miRNA concentrations were log-transformed in models due to skewness and are presented as geometric mean (95% confidence interval). Fold

difference of the PCR-based miRNA concentrations was the fold difference of the geometric mean. Spearman correlation was used to assess the correlation of continuous variables.

Sample size determination

For sRNA sequencing the discovery cohort sample size of 167 RA and 91 control subjects offered approximately 99% power to detect miRNAs which were >1.5 fold altered in RA versus control subjects assuming detection across all samples of approximately 500 miRNAs with a 5% false discovery rate (https://cqs.mc.vanderbilt.edu/shiny/RnaSeqSampleSize/). This sample size also gave approximately 99% power to detect an AUC ≥0.65 based on PCR-based plasma miRNA concentrations.

For the validation cohort, a sample size of 32 patients with RA and 32 controls provided approximately 90% power to detect and AUC ≥0.7 based on PCR-based plasma miRNA concentrations.

Pathway analysis

We separately evaluated upstream regulators of the panel miRNAs and downstream targets regulated by the panel miRNAs using Ingenuity Pathway Analysis (Qiagen, Version 01-07). For evaluation as upstream regulators, we selected direct and indirect regulators of each mature miRNA and its precursor using all available data. For evaluation of downstream targets of the panel miRNAs, a target was included in analysis if it was previously experimentally validated or is a highly predicted target of the miRNA. We assessed canonical pathways and functional analyses.

RESULTS

Clinical characteristics

The discovery cohort included 167 patients with RA and 91 control subjects. The groups were of similar age, race and sex (Table 1). The RA validation cohort included 32 patients with RA and 32 control subjects of similar age, race and sex (Supplemental Table 1). Compared to the discovery cohort disease activity was lower (DAS28 score 3.89 vs 2.80 units), and there were fewer seropositive individuals in the validation cohort (69% vs 54% positive for rheumatoid factor). The SLE validation cohort included 12 patients with SLE (Supplemental Table 2).

Significantly altered miRNAs based on sRNA sequencing comparing RA vs control subjects – discovery cohort

Among the 262 reliably detected plasma miRNAs, 175 were significantly altered in RA compared to control subjects after adjusting for age, race, sex and batch and FDR. Among these, 110 were ≥1.5 fold altered in RA compared to control subjects (Figure 1). Most of these miRNAs were increased in RA plasma, and one miRNA, miR-3168, was significantly decreased (−1.73-fold decreased, P=5.0E-03, Padj=1.2E-02).

The top 15 differentially expressed plasma miRNAs as determined each by DESeq2 and by random forest analysis are listed in Table 2. Twelve of the 15 miRNAs were common to both

analytic approaches (miR-3615, miR-22-3p, miR-502-3p, miR-345-5p, miR-29c-3p, miR-221-3p, miR-140-3p, miR-30e-5p, miR-501-3p, miR-22-5p, miR-127-3p, miR-134-5p). Additionally, 3 miRNAs were identified each by DESeq2 only (miR-99b-5p, miR-130a-3p, miR-21-3p), and 3 by random forest only (miR-627-5p, miR-24-3p, miR-96-5p).

PCR validation of altered miRNAs

The top candidate miRNAs (18 total) were measured by qPCR in the discovery cohort. Two of the miRNAs (miR-502-3p and miR-501-3p) were too low in abundance to be assayed reliably by qPCR. All but two (miR-127-3p and miR-96-5p) of the remaining miRNAs were significantly increased among patients with RA compared to control subjects using PCR-based concentrations of miRNAs (Table 2).

miRNA panel development

Using lasso variable selection with logistic regression of the qPCR-based concentrations of miRNAs in the discovery cohort, the following miRNAs were chosen for the panel so as to include the fewest miRNAs that discriminated RA from control subjects: miR-22-3p, miR-24-3p, miR-96-5p, miR-134-5p, miR-140-3p, miR-627-5p. The panel had an AUC=0.81 (95% CI: 0.75, 0.87; P<0.001) for differentiating RA and control subjects. The panel was similarly robust among those with seropositive RA AUC=0.79 (95% CI: 0.73, 0.86; P<0.001), seronegative RA AUC=0.84 (95% CI: 0.77, 0.91; P<0.001), RA in remission (DAS28 score<2.6(25)) (AUC=0.85 (95% CI: 0.78, 0.92; P<0.001) and high RA disease activity (DAS28>5.1) (AUC=0.79 (95% CI: 0.70, 0.88; P<0.001).

The panel had similar performance across other subgroups of patients compared to control subjects. This included: RA patients with disease duration <1 year (N=29) AUC=0.80 (95% CI: 0.72, 0.89; P<0.001), those not taking biologic DMARDs (bDMARDs) (N=133) AUC=0.81 (95% CI: 0.75, 0.87; P<0.001), those taking bDMARDs (N=33) AUC=0.80 (95%CI: 0.71, 0.88; P<0.001), those not taking conventional synthetic DMARDs (csDMARDs) or bDMARD (N=19) AUC=0.90 (95%CI: 0.83, 0.96; P<0.001), those taking any csDMARD or bDMARD (N=150) AUC=0.80 (0.73, 0.86; P<0.001), those not receiving any csDMARD, bDMARD or corticosteroid (N= 13) AUC= 0.89 (95%CI: 0.83, 0.96; P<0.001), and those taking either csDMARD or bDMARD or corticosteroid only (N=153) compared to control subjects AUC=0.80 (95% CI: 0.74, 0.86; P<0.001).

Validation

The panel of six miRNAs was robust in the separate RA validation cohort (AUC=0.71) (95% CI: 0.58, 0.84; P=0.004). Similarly, in the validation cohort, the panel differentiated between seropositive (AUC=0.73; 95% CI: 0.58, 0.87; P=0.01) or seronegative RA (AUC=0.73; 95% CI: 0.57, 0.89; P=0.02) vs control subjects.

We additionally measured the panel in 12 patients with SLE and compared model performance to control subjects. The panel also differentiated SLE patients from controls subjects (AUC=0.80 (95% CI: 0.65, 0.96; P=0.001)), but was not significantly different comparing patients with SLE to RA (AUC=0.63 (95% CI: 0.44, 0.82; P=0.13)).

Relationship between miRNA components of the panel and disease-related variables

Three of the miRNAs were weakly associated with RA disease activity by DAS28 score in the discovery cohort (miR-24-3p: Rho= −0.16, P=0.04; miR-96-5p: Rho= 0.16, P=0.04; miR-140-3p: Rho=−0.16, P=0.05); however, these significant associations were not observed in the RA validation cohort.

What are the upstream regulators of these miRNAs in the panel?

We examined upstream regulators of the miRNAs included in the panel to determine if there are commonalities in regulation between the miRNAs which would promote their ability to be used as a RA miRNA signature. There was little information regarding upstream regulators of miR-627-5p or its precursor at the time of analysis, thus this miRNA was excluded from pathway analysis. The top identified function that the upstream regulators possess related to invasion of cells (P=7.89E-27), for which 35 of the upstream regulators were included (Figure 2). Additionally, these upstream regulators are involved in cell death (P=7.11E-22). The top overlapping canonical pathway was role of macrophages, fibroblasts and endothelial cells in RA with 11 overlapping molecules (Figure 2).

miRNA panel pathway targets

Because circulating miRNAs can be delivered to cells with functional consequences (5) or could reflect cellular processes, we evaluated if experimentally validated and highly predicted targets of panel miRNAs are involved in RA pathways. There was little information regarding miR-134-5p function at the time of analysis, thus this miRNA was excluded from pathway analysis. Among the top canonical pathways, role of osteoblasts, osteoclasts and chondrocytes in RA (Supplemental Figure) and role of macrophages, fibroblasts and endothelial cells in RA (Figure 3) were third (29 molecules) and sixth (28 molecules) respectively in the number of molecules in the pathway which the miRNA panel may target. Other top canonical pathways include molecular mechanisms of cancer (52 genes), G-protein coupled receptor signaling (30 molecules), protein kinase A signaling (29 molecules) and axonal guidance signaling (28 molecules).

DISCUSSION

We used plasma sRNA sequencing and bioinformatics approaches to develop a panel of miRNAs that reliably differentiates between patients with RA and control subjects. The panel included miR-22-3p, miR-24-3p, miR-96-5p, miR-134-5p, miR-140-3p, and miR-627-5p and was robust across seronegative and seropositive RA and RA of varying disease activity. The panel also differentiated between patients with SLE and control subjects but was not significantly different between patients with RA and SLE, suggesting that this panel represents an autoimmunity signature.

Strong evidence indicates that miRNAs as a class of sRNAs are important in RA. Dicer and Drosha, which are endoribonucleases involved in cleaving miRNAs to their mature form, are upregulated in peripheral blood mononuclear cells (PBMCs) of patients with RA (26). Moreover, activation of Dicer decreases tumor necrosis factor alpha (TNFα) production, suggesting a homeostatic role of miRNAs in RA. The upregulation of Dicer and Drosha in

PBMCs from patients with RA likely explains why we observed an overall increase in miRNAs in plasma from patients with RA, since many plasma miRNAs derive from PBMCs and other blood cells (27). However, in RA not all cell types have increased expression of Dicer and the associated increased miRNA expression. For example, Dicer and miRNA expression were lower in RA synovial fibroblasts, leading to an exaggerated response to inflammatory stimuli and resistance to apoptosis (28). In addition to broad changes in miRNA production in RA, there are several individual miRNAs with biologic significance implicated in RA.

Several of the panel miRNAs have known associations with RA, either as biomarkers or known biology of RA. For example, miR-22-3p was increased significantly among ACPA positive individuals who subsequently progressed to RA (29). Also, lower concentrations of miR-22-3p before treatment were associated with response to adalimumab at 12 months in a randomized double-blinded placebo-controlled trial of 180 patients with early RA (30). miR-22-3p is decreased in fibroblast-like synoviocytes (FLS) from RA compared to osteoarthritis patients; lower concentrations of miR-22-3p promoted FLS proliferation (31). Thus miR-22-3p may be a driving force for the synovial hyperplasia characteristic of RA. miR-22-3p also regulates Th17 responses in emphysema (32) and drives hyperresponsive B cells in SLE (33). Moreover, we found that inhibition of miR-22-3p improves nephritis in a mouse model of SLE (*manuscript In preparation*).

Plasma concentrations of miR-24-3p were elevated in patients with RA compared to controls in several prior studies, and is a component of a prior RA plasma miRNA panel which we and others have proposed (12, 14). In response to IL-6, miR-24-3p increases and promotes plasma cell survival, an effect that could support autoreactive plasma cells in RA (34). miR-24-3p also plays a homeostatic role by dampening inflammation through nuclear factor-KB signaling pathway (35) and chitinase 3-like-1 (36) in the vasculature.

miR-140-3p has been most widely studied in osteoarthritis and participates in cartilage homeostasis (37); its concentrations are also decreased in synovial tissue from patients with RA compared to osteoarthritis (38). Intra-articular administration of pre-miR-140 (the precursor for miR-140-3p) reduced arthritis scores in mice with collagen induced arthritis by way of increasing synovial fibroblast apoptosis and reducing proliferation (38).

Three of the panel miRNAs (miR-96-5p, miR-134-5p, and miR-627-5p) have not been studied widely in RA. Thus, if we had limited selection of miRNAs for the panel to those previously studied in RA, these would have been overlooked. We believe we identified new miRNAs because of our methods which used sequencing rather than a preselected panel and random forest analysis to identify candidates, which has not been previously done in RA to our knowledge. miR-96-5p remained helpful to the model despite not being overall significantly altered in RA based on PCR data. Both miR-96-5p and miR-134-5p can target the KRAS signaling pathway (39, 40), which may affect T-cell activation thresholds, enabling responses to autoantigens (41). Calcitriol induced expression of miR-627 and miR-627 reduced proliferation of colorectal cancer cell line (42). Exposure of calcitrol to synoviocytes cultured from RA patients reduced cell proliferation and cytokine production (43). It is possible that miR-627 may be part of this mechanism.

Proposed pathways which promote or are altered by the miRNAs In the panel

Pathway analysis indicates that upstream regulators of panel miRNAs and that panel miRNA targets are involved in RA-related disease processes. This observation also provides proof-of-principle for the methodology we used to develop the panel. Use of non-biased techniques, such as random forest analysis, serves to find novel biomarkers and offer some insight into mechanisms of disease.

Limitations

This study had some limitations. Both discovery and validation cohorts were predominantly patients with established disease (defined as ≥6 months duration (44)); thus, the panel may not differentiate individuals with RA at the time of diagnosis or pre-RA states from control subjects. The panel was robust among those with disease duration <1 year and among those not on DMARDs, however. We did not design the study to develop a diagnostic panel for RA when other inflammatory autoimmune diseases are under consideration, but initial testing of the panel in a small set of patients with SLE suggests that the panel may represent an autoimmunity signature. Future studies examining the panel in a variety of inflammatory autoimmune diseases at varied levels of activity would be helpful to determine if these miRNAs compose an inflammatory autoimmune disease signature rather than an RA signature. The strong relationship between the miRNAs and annotated RA pathways was reassuring, but there are limitations to pathway analysis. In general RA has more extensive annotated pathways than many other inflammatory autoimmune diseases, so it is possible that we do not see as strong a relationship with other inflammatory autoimmune diseases because there are insufficient annotated pathways.

Conclusion

A miRNA panel identified by bioinformatics approaches was able to differentiate between patients with RA and control subjects with reproducibility. Many of the upstream regulators of the miRNAs and many of the miRNA targets regulate RA-related pathways. The panel may represent an autoimmunity signature which is not dependent on active disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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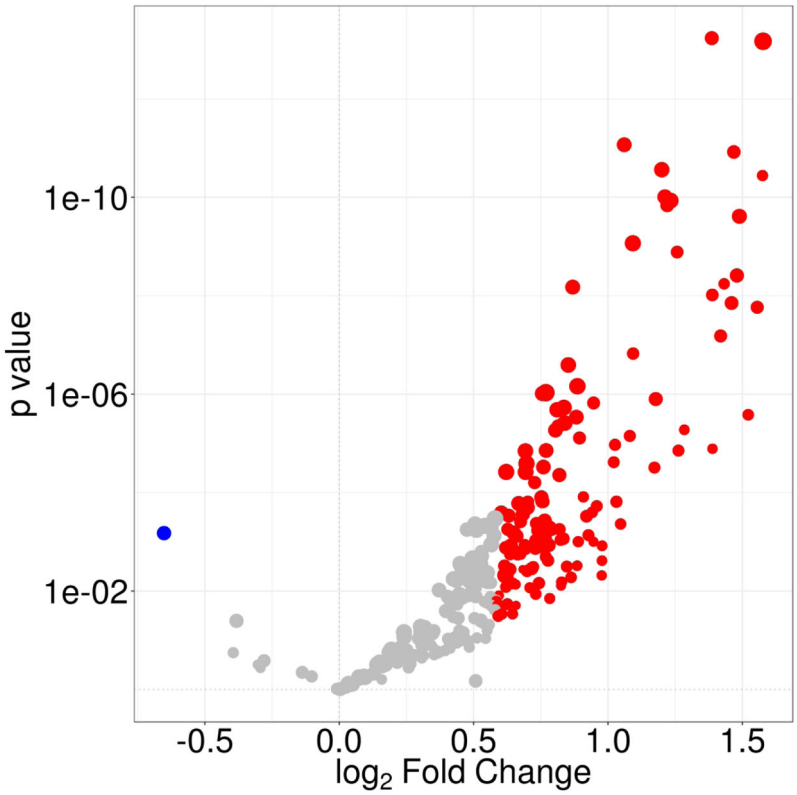

Figure 1.

Volcano plot displaying differential plasma miRNA expression in the discovery cohort (n=167 RA vs n=91 control subjects). Small RNA sequencing was analyses by DESeq2 and adjusted for age, race, sex and batch and multiple comparisons. Among these 110 were ≥1.5 fold altered in RA compared to control subjects. Each dot represents an individual miRNA and the larger the dot the more abundant the miRNA is. Red indicates increased in RA and blue indicates increased in control subjects.

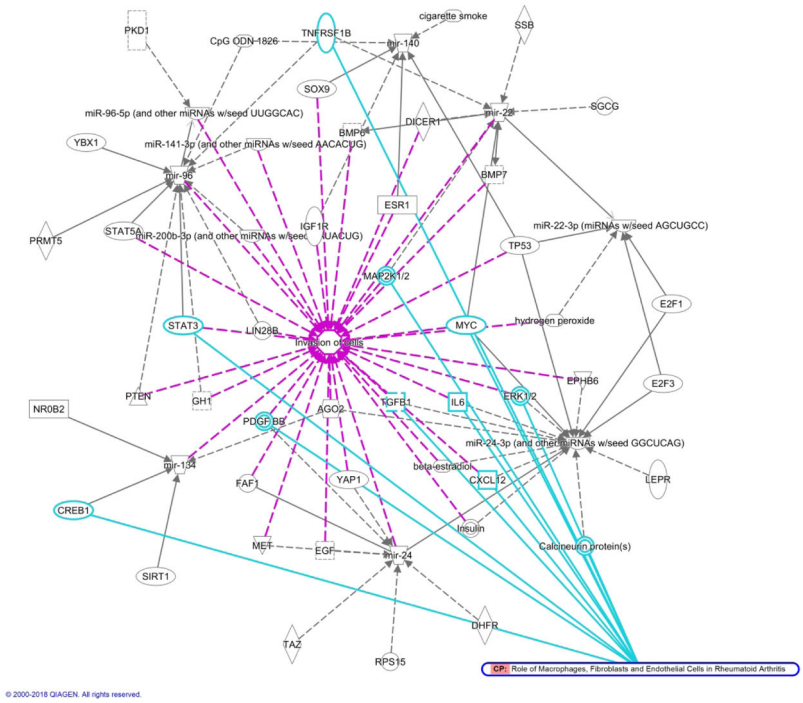

Figure 2.

Upstream regulators of RA panel miRNAs are involved in invasion of cells and RA-specific pathways. Using Ingenuity Pathway Analysis of upstream regulators of the mature and precursor miRNAs, the top related functional pathway was related to invasion of cells and the top related canonical pathway was role of macrophages, fibroblasts, and endothelial cells in RA.

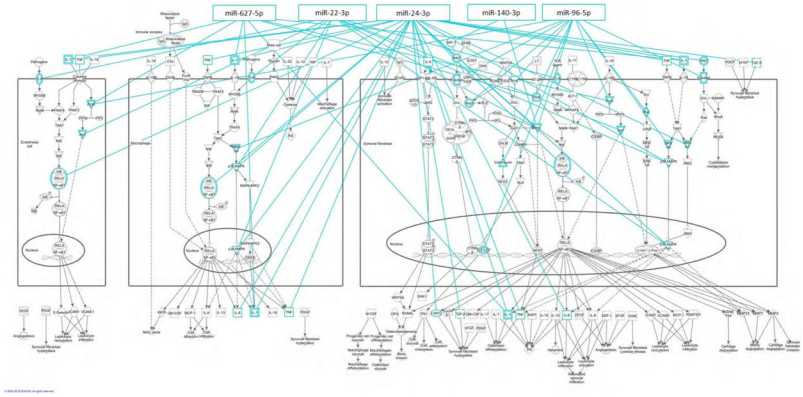

Figure 3.

RA panel miRNAs can target RA-specific pathways. Using Ingenuity Pathway Analysis of experimentally validated or highly predicted targets of the panel miRNAs, one of the top related canonical pathways was role of macrophages, fibroblasts, and endothelial cells in RA.

Table 1.

Subject characteristics- discovery cohort

	RA (N=167)	Control (N=91)	P
Age, years	54 [45, 63]	53 [44, 59]	0.35
Race, #Caucasian	148 (89)	77 (85)	0.49
Sex, #female	114 (68)	57 (63)	0.36
Disease duration, years	3 [2, 18]	-	-
DAS28 score, units	3.89 [2.63, 4.9]	-	-
Tender joints, #	2 [0, 7]	-	-
Swollen joints, #	3 [0, 8]	-	-
ESR, mm/hr	16 [7, 36]	-	-
hsCRP, mg/L	4 [1.2, 11.0]	0.5 [0.2, 1.7]	<0.001
RF positive, #	115 (69)	-	-
Methotrexate, #	117 (71)	-	-
Leflunomide, #	29 (17)	-	-
Hydroxychloroquine, #	42 (25)	-	-
Corticosteroids, #	89 (53)	2 (2)	<0.001
Anti-TNF, #	33 (20)	-	-

Data are presented as median [interquartile range] or number (%). DAS28=disease activity based on 28 joints using erythrocyte sedimentation rate, ESR=erythrocyte sedimentation rate, hsCRP= high sensitivity C-reactive protein, RF= rheumatoid factor (data available on 28 patients with RA). CCP= anti-cyclic citrullinated peptide antibody (data available on 17 patients with RA).

Table 2.

PCR validation of top miRNA candidates from discovery cohort sRNA sequencing

	Plasma fM concentration presented as geometric mean (95% CI)			
	RA	**Control**	**Fold diff**	**P**
miR-3615	0.34 (0.28, 0.41)	0.25 (0.17, 0.35)	1.38	4.69E-01
miR-22-3p	58.1 (45.3, 74.5)	15.1 (10.8, 21)	3.85	8.45E-12
miR-502-3p	N/A	N/A	N/A	N/A
miR-345-5p	3.08E-2 (1.63E-02, 5.81E-02)	7.75E-3 (2.95E-03, 2.04E-02)	3.98	1.72E-04
miR-29c-3p	1.46 (1.18, 1.8)	0.56 (0.41, 0.77)	2.59	5.32E-09
miR-99b-5p	1.50 (0.86, 2.61)	0.99 (0.48, 2.05)	1.51	7.27E-04
miR-221-3p	9.96 (7.48, 13.2)	3.04 (2.15, 4.31)	3.27	6.78E-09
miR-140-3p	1.22 (1.0, 1.49)	0.25 (0.17, 0.37)	4.93	2.12E-13
miR-130a-3p	5.00 (3.51, 7.12)	1.27 (0.81, 1.97)	3.94	8.56E-08
miR-30e-5p	4.23 (3.51, 5.11)	3.24 (2.49, 4.21)	1.31	2.39E-02
miR-501-3p	N/A	N/A	N/A	N/A
miR-22-5p	1.18 (0.891, 1.56)	0.39 (0.25, 0.61)	2.99	1.25E-07
miR-127-3p	5.17 (4.7, 5.68)	5.53 (4.9, 6.24)	−1.08	4.88E-01
miR-21-3p	1.21E-02 (4.56E-03, 3.23E-02)	6.97E-04 (1.33E-04, 6.64E-03)	17.4	4.08E-03
miR-134-5p	1.45E-01 (6.38E-02, 3.35E-01)	6.76E-03 (1.64E-03, 2.80E-02)	21.6	5.11E-07
miR-627-5p	7.18E-02 (2.99E-02, 1.72E-01)	4.39E-03 (9.58E-04, 2.01E-02)	16.3	2.73E-04
miR-24-3p	3.49 (2.35, 5.18)	1.32 (0.85, 2.06)	2.64	1.19E-06
miR-96-5p	13.9 (12.9, 14.9)	15.2 (13.6, 17)	−1.10	1.85E-01

N/A indicates that the miRNA was too low for reliable detection by PCR. fM=femtomolar concentration. Fold diff= fold difference comparing the geometric mean of qPCR-based miRNA concentration in RA vs control subjects.