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Circulating Small Noncoding RNA Biomarkers of Response to Triple Disease-modifying Antirheumatic Drug Therapy in White Women With Early Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To identify small noncoding RNA (sncRNA) serum biomarkers that predict response to triple disease-modifying antirheumatic drug (DMARD) therapy in patients with early rheumatoid arthritis (RA).
Methods. Early RA patients entered into a treat-to-target management algorithm, with triple DMARD therapy (methotrexate, sulfasalazine, hydroxychloroquine). Patients were assessed following 6 months of therapy and classified as European League Against Rheumatism responders or nonresponders. RNA was isolated from 42 archived serum samples, collected prior to commencement of triple DMARD therapy. Small RNA sequencing was performed and the reads mapped to annotations in a database of human sncRNA. Differential expression analysis was performed, comparing responders (n = 24) and nonresponders (n = 18).
Results. Pretreatment levels of 4 sncRNA were significantly increased in nonresponders: chr1.tRNA131-GlyCCC (4.1-fold, adjusted P = 0.01), chr2.tRNA13-AlaCGC (2.2-fold, adjusted P = 0.02), U2-L166 (6.6-fold, adjusted P = 0.02), and piR-35982 (2.4-fold, adjusted P = 0.03). 5S-L612 was the only sncRNA significantly increased in responders (3.3-fold; adjusted P = 0.01). Reads for chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC mapped to the 5′ end of each tRNA gene and were truncated at the anticodon loop, consistent with these sncRNA having roles as 5′ translation interfering tRNA halves (tiRNA).
Conclusion. Pretreatment levels of specific serum sncRNA might facilitate identification of patients more likely to respond to triple DMARD therapy.

Key indexing terms: biomarker, DMARD, rheumatoid arthritis, small noncoding RNA, translation interfering tRNA halves

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Blood-based biomarkers can help guide therapy, avoid invasive tissue biopsy, and provide insight into the underlying pathophysiology of chronic inflammatory diseases, such as rheumatoid arthritis (RA). Circulating extracellular small noncoding RNA (sncRNA) have shown promise as novel biomarkers for clinical application[1,2]. sncRNA include microRNA (miRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), piwi-interacting RNA (piRNA), and small nuclear RNA (snRNA). These sncRNA are attractive as biomarkers due to abundance, stability, and potential to provide information on underlying disease processes[1].

Triple disease-modifying antirheumatic drug (DMARD) therapy with methotrexate (MTX), sulfasalazine, and hydroxychloroquine is an effective treatment regimen for RA[3]. The efficacy and relative low cost of triple DMARD therapy has led to its uptake as a first-line therapeutic approach for RA. However, a significant proportion of patients do not respond[4,5]. As a result, these patients can experience disease progression. Currently, there is no way of determining whether an individual is likely to benefit from triple DMARD therapy or would be better managed with alternative treatment from the outset.

A few reports document baseline expression of circulating miRNA as potential indicators of response to various treatment

regimens in RA, mostly focused on the response to biologic agents[6,7,8,9,10,11]. These studies have been hindered by small patient cohorts and loss of significance following correction for multiple testing. Further, most previous observations have not been validated in independent cohorts.

In this study, using small RNA sequencing (RNAseq), sncRNA expression was quantified in serum samples collected from patients newly diagnosed with RA, prior to the commencement of triple DMARD therapy. Following adjustment for multiple testing, 5 sncRNA were identified that were differentially expressed in patients who subsequently manifested good or nonresponsiveness to triple DMARD therapy.

MATERIALS AND METHODS

Participants. Archived serum samples were obtained from DMARD-naïve patients attending the Early Arthritis Clinic at Royal Adelaide Hospital. These patients met the inclusion criteria for active RA based on the American College of Rheumatology 1987 revised criteria and had disease duration of < 12 months. Patient details and clinical variables are specified in Table 1. To limit variability unrelated to DMARD responsiveness, we restricted our analysis to white women. Serum was aliquoted upon collection and stored at –80°C. All participants subsequently received treatment in accordance with a standardized triple DMARD therapeutic regimen, as previously described[12]. Patients were evaluated after 6 months of therapy and classified as European League Against Rheumatism responders (i.e., good or moderate) or nonresponders[13]. The study was approved by Melbourne Health Research and Ethics Committee (2010.293), and by Royal Adelaide Hospital and The Walter and Eliza Hall Institute human research ethics committees.

RNA isolation, sequencing, differential expression, and bioinformatic analysis. RNA was isolated from serum using the Norgen Plasma/Serum Circulating and Exosomal RNA Purification Kit, and cDNA libraries were prepared, uniquely barcoded, and sequenced using an Illumina NextSeq 500. Raw sequencing data has been uploaded to the NCBI Sequence Read Archive (PRJNA602835). Analysis of RNAseq data was performed using R Statistical software v3.4.2 (R Foundation for Statistical Computing).

Table 1. Patient demographics and baseline clinical variables.

	Responders	Nonresponders	*P*
N	24	18	1
Females, %	100	100	1
Age, yrs	60.4 (14.5)	54.6 (11.5)	0.782
Weeks of polyarthritis	23.9 (19.0)	40.2 (69.5)	0.432
ACPA, % positive	75	52.9	0.189
Rheumatoid factor, % positive	91.7	44.4	*0.001*
DAS28-ESR	5.7 (1.1)	5.6 (1.3)	0.997
Swollen 28-joint count	9.7 (5.7)	10.0 (7.5)	0.989
Tender 28-joint count	11.9 (6.5)	15.5 (8.6)	0.863
CRP, mg/L	25.5 (22.8)	13.3 (21.1)	0.557
ESR, mm/h	39.5 (30.7)	25.3 (15.0)	0.496
White cell count, 1×10^6 cells/mL	8.6 (2.6)	7.4 (3.8)	0.956

Values are expressed as mean (SD) unless otherwise indicated. ACPA and RF positivity analyzed with Fisher exact test. All other variables analyzed with Student *t* test. ACPA: anticitrullinated protein antibody; CRP: C-reactive protein; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor.

Reads were aligned to the human genome hg19, then mapped to annotations within the database of small human noncoding RNA (DASHR)[14]. Any RNA that did not achieve a count per million of mapped reads > 1 in at least 12 samples was filtered out from the data, leaving 1487 sncRNA for analysis. Hierarchical clustering and principal component analysis for all 1487 snRNA analyzed are included in Supplementary Figure 1 (available with the online version of this article). Differential expression analysis was performed using the edgeR and limma software packages. Correlations between RNA expression and Disease Activity Score in 28 joints based on erythrocyte sedimentation rate (ESR) values were calculated using edgeR's likelihood ratio test. *P* values were adjusted using the Benjamini-Hochberg method. Read counts, normalization factors, and proportions of sncRNA biotypes for each sample are specified in Supplementary Table 1 and Supplementary Figure 2. Additional methodological details are located in online Supplementary Data 1.

RESULTS

Five sncRNA are differentially expressed at baseline between responders and nonresponders. To identify sncRNA with expression indicative of subsequent triple DMARD responsiveness, small RNAseq and differential expression analysis was performed on serum obtained from a cohort of early RA patients, prior to commencing triple DMARD therapy. Five sncRNA were identified with an adjusted *P* value (adj *P*) < 0.05 (Supplementary Table 2, available with the online version of this article). These consisted of 2 tRNA, chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC; a 5S rRNA, 5S-L612; an snRNA, U2-L166; and a piRNA, piR-35982 (Figure 1A). Of these, 5S-L612 was elevated in responders, whereas chr1.tRNA131-GlyCCC, chr2.tRNA13-AlaCGC, U2-L166, and piR-35982 were elevated in nonresponders (Figure 1B,C). Analysis details for all 1487 detected sncRNA are specified in Supplementary Table 2.

5′ tRNA halves are enriched in nonresponders to triple DMARD therapy. Because circulating tRNA fragments are elevated in various diseases[15], we investigated whether sequencing reads attributed to the 2 differentially expressed tRNA wholly or partially aligned to their respective full-length tRNA genes. Strikingly, all reads attributed to chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC only partially aligned to the full-length gene sequences (Figure 2A). Further, the majority of reads were derived from the 5′ end of the tRNA gene and were truncated at the anticodon loop (Figure 2B), consistent with 5′ translation interfering tRNA halves (tiRNA)[15].

sncRNA associations with clinical variables. To explore circulating sncRNA as potential biomarkers of disease activity, associations between sncRNA expression and clinical variables were investigated (Supplementary Tables 3–12, available with the online version of this article). chr1.tRNA131-GlyCCC expression was significantly decreased in patients positive for anticitrullinated protein antibody (ACPA) (2.26 $\log_2$-fold; adj $P = 0.0497$) and rheumatoid factor (RF; chr1.tRNA131-GlyCCC (2.57 $\log_2$-fold; adj $P = 7.15e\text{-}5$). A significant positive correlation with tender 28-joint count (TJC28; r = 0.14, adj $P = 7.19e\text{-}6$) was also apparent. piR-35982 was significantly decreased in patients positive for RF (1.36 $\log_2$-fold; adj $P = 0.049$) and showed modest, negative correlations with C-reactive protein

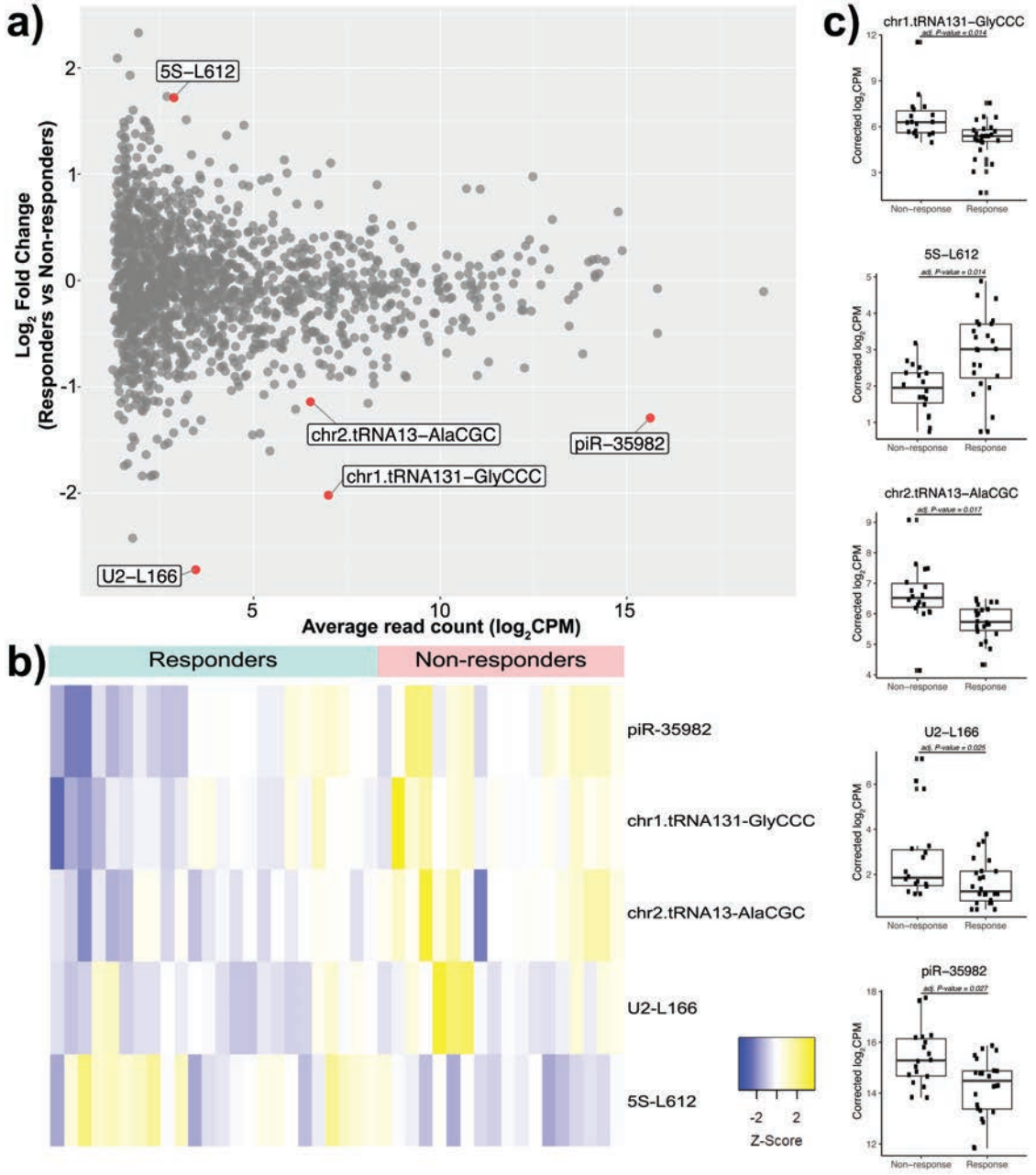

Figure 1. sncRNA differentially expressed between responders and nonresponders. (a) MA plot of counts per million reads (CPM) vs fold change for all 1487 sncRNA analyzed. sncRNA with an adjusted *P* value < 0.05 are indicated in red and labeled. (b) Supervised heatmap showing relative expression of significantly differentially expressed sncRNA across individual patient samples. Heatmap rows show differentially expressed sncRNA. Heatmap columns represent individual patients. Relative RNA abundance is coloured by Z score as indicated. (c) Box and whisker plots comparing expression and variability of differentially expressed sncRNA. Lower and upper hinges respectively correspond to the first and third quartiles. Whiskers extend from hinges to highest and lowest values that are within 1.5 × IQR. Mean values are indicated.

(r = –0.03, adj P = 0.03) and ESR (r = –0.06, adj *P* = 0.03). U2-L166 showed a positive correlation with TJC28 (r = 0.20, adj *P* = 0.03).

DISCUSSION

RA is a complex, heterogeneous inflammatory disease. Currently, there is no way of prospectively determining the optimal therapeutic approach for an individual who is newly diagnosed with RA. Biomarkers that enabled rational selection of therapy at the time of diagnosis would help stratify management of patients with RA. In this study, we identified 5 circulating sncRNA that are differentially expressed between responders

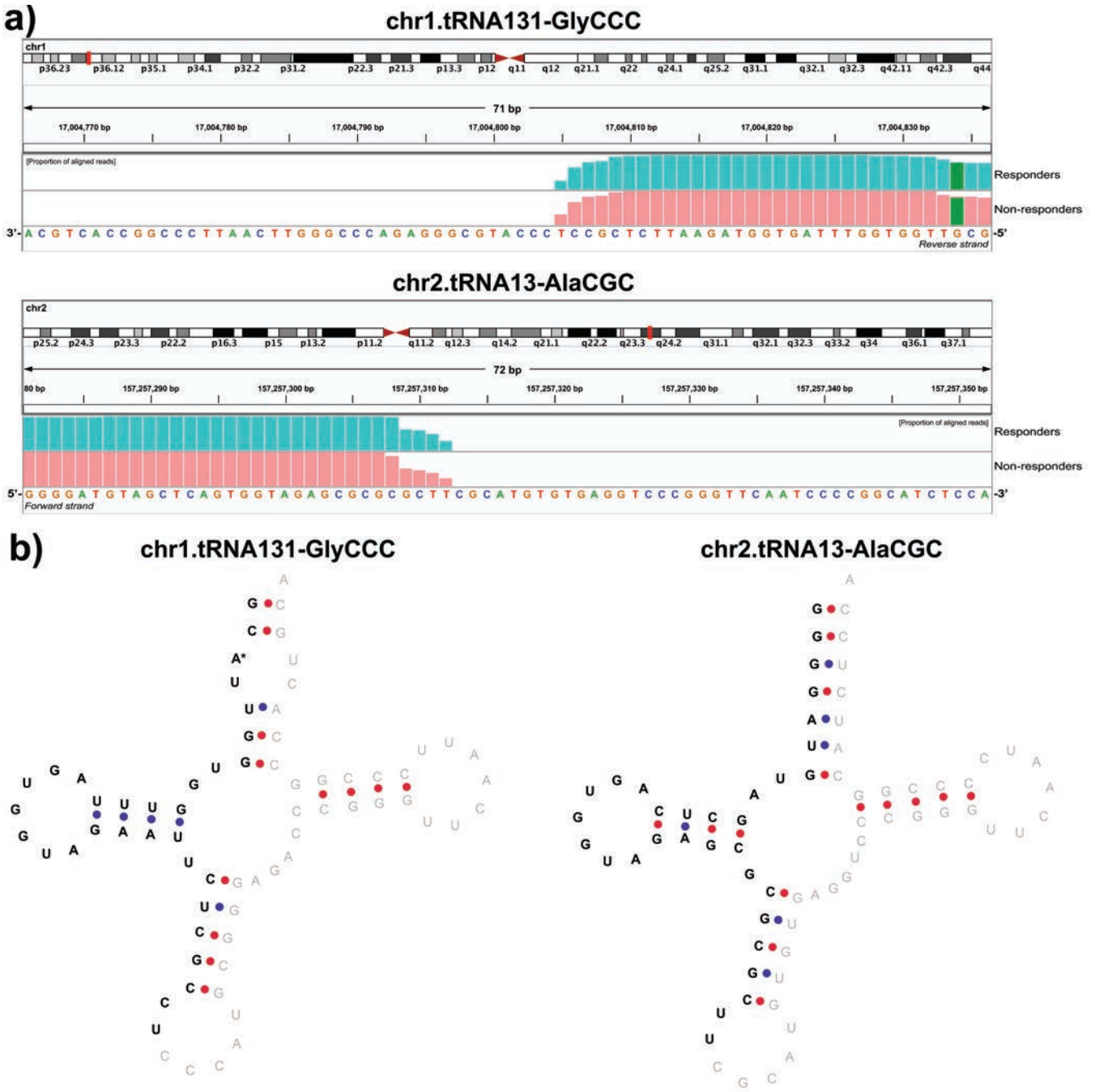

Figure 2. tRNA reads are truncated at the anticodon loop. (a) Alignments of chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC reads against their full-length gene sequence. Chromosomal locations of genes are indicated, and full-length canonical gene sequences are specified. Nucleotide variations from canonical sequence are colored accordingly. (b) Position of sequence reads in relation to tRNA secondary structures. For both tRNA, reads are attenuated at the anticodon loop. Nucleotides within sequencing reads are indicated in black; absent nucleotides are indicated in gray. Nucleotide variations from canonical gene sequence are specified with an asterisk.

and nonresponders to triple DMARD therapy. If confirmed, these circulating sncRNA could help guide rational selection of therapy and provide further information on the underlying biology of treatment responses.

Understanding the physiological functions of sncRNA in RA and/or DMARD pharmacology might also offer new insights into disease. We found chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC 5′ tiRNA were significantly elevated in nonresponders to triple DMARD therapy. tiRNA are evolutionary conserved RNA that are produced following cleavage of full-length tRNA at the anticodon loop by the RNase angiogenin[15]. Fragmentation of tRNA extends tRNA function

beyond facilitating protein synthesis[15], with tiRNA having roles in inhibiting protein synthesis, inhibiting apoptosis, and sequestering proteins[15]. A recent study reported that a 5′-tiRNA-Ala mimic similar to chr2.tRNA13-AlaCGC tiRNA enhanced the migratory and invasive capacity of HCT116 colorectal cancer cells[16]. Although the biological mechanism underlying this function is unknown, it is conceivable that chr2.tRNA13-AlaCGC might similarly contribute to proinflammatory cell behaviour in RA.

sncRNA are present at high levels in the circulation and form part of ribonucleoprotein complexes, or are associated with high-density lipoproteins or extracellular vesicles[1]. Encapsulation or association of sncRNA with extracellular vesicles, high-density lipoproteins, and ribonucleoprotein complexes can facilitate RNA uptake and thereby the functional modification of target cells[1]. In this way, circulating sncRNA might be able to regulate cellular physiology. It remains to be determined whether the differentially expressed sncRNA identified in our study have biological function or represent metabolic by-products.

Of note, our results conflict with an earlier report that found expression of miR-16 and miR-223 were increased in the serum of RA patients who responded to conventional DMARD[10]. These miRNA were not identified as significantly differentially expressed in our study. Likewise, circulating miRNAs previously reported as correlating with disease activity[10,17,18] were not confirmed in our study. These differences might be explained by variations in methodology, plus our use of correction for multiple testing, which is especially important for high-throughput RNAseq analyses.

To be of clinical use, biomarkers must have high specificity. Importantly, RA patients with more severe disease are known to be less likely to respond to treatment[19]. To this end, sncRNA associated with response to therapy must be distinguished from sncRNA that are associated with more severe disease. We found expression of chr1.tRNA131-GlyCCC 5′ tiRNA was significantly increased in nonresponders to triple DMARD therapy, with the lowest *P* value of all sncRNA analyzed. Consistent with poorer treatment outcomes for patients negative for ACPA and RF[20], levels of chr1.tRNA131-GlyCCC were significantly increased in these patients. Further, a significant positive correlation between chr1.tRNA131-GlyCCC levels and TJC28 scores ($r = 0.14$, adj $P = 7.19e\text{-}6$) suggests that elevated circulating chr1.tRNA131-GlyCCC 5′ tiRNA levels might signify increased inflammation. Weaker associations between 5S-L612, chr2.tRNA13-AlaCGC, U2-L166, and piR-35982 expression and clinical variables suggest these sncRNA are more likely to reflect DMARD responsiveness.

In summary, we identified 5 circulating sncRNA in patients with early RA that might represent clinically useful biomarkers of subsequent triple DMARD responsiveness. In addition, sncRNA such as tiRNA may play an unappreciated role in the pathogenesis of RA or in the response to treatment. Major strengths of this study include the serial evaluation of a patient cohort treated in a uniform manner and the relatively homogeneous patient group. Limitations include the need for validation in an independent cohort and the relatively small sample size of exclusively white women. We also found a significant difference in RF positivity between responders and nonresponders in our patient cohort. While RF status is reported to be a poor predictor of MTX response[21], its ability to predict the response to triple DMARD has not been extensively investigated. The sncRNA identified in this study may provide new insights into the pathogenesis and treatment of RA. Our findings suggest further study of this class of potential biomarkers is warranted in RA.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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