Circulating small non-coding RNA biomarkers of response to triple DMARD therapy in Caucasian women with early rheumatoid arthritis

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Short running head:

sncRNAs predict DMARD response

Abstract

Objective

To identify small non-coding 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 + sulphasalazine + hydroxychloroquine). Patients were assessed following 6 months of therapy and classified as EULAR responders or non-responders. 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 small human non-coding RNAs. Differential expression analysis was performed, comparing responders (n=24) and non-responders (n=18).

Results

Pre-treatment levels of 4 sncRNAs were significantly increased in non-responders: chr1.tRNA131-GlyCCC (4.1-fold, adj.P-value: 0.01); chr2.tRNA13-AlaCGC (2.2-fold, adj.P-value:0.02); U2-L166 (6.6-fold, adj.P-value: 0.02); and piR-35982 (2.4-fold; adj.P-value: 0.03). 5S-L612 was the only sncRNA significantly increased in responders (3.3-fold; adj.P-value: 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 anti-codon loop, consistent with these sncRNAs having roles as 5' translation interfering tRNA halves (tiRNAs).

Conclusion

Pre-treatment levels of specific serum sncRNAs might facilitate identification of patients more likely to respond to triple DMARD therapy.

Introduction

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 non-coding RNAs (sncRNAs) have shown promise as novel biomarkers for clinical application[1,2]. sncRNAs include microRNA (miRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), piwi-interacting RNA (piRNA) and small nuclear RNA (snRNA). These sncRNAs are attractive as biomarkers due to abundance, stability and potential to provide information on underlying disease processes[1].

Triple DMARD therapy with methotrexate, sulphasalazine and hydroxychloroquine is an effective treatment regimen for RA[3]. The efficacy and relative inexpense 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 miRNAs as potential indicators of response to various treatment regimens in RA, mostly focussed on the response to biologic agents[6-11]. These studies have been hindered by small patient cohorts and loss of significance following correction for multiple testing. Furthermore, 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, five sncRNAs were identified that were differentially expressed in patients who subsequently manifested good or non-responsiveness 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 parameters are specified in **Table 1**. To limit variability unrelated to DMARD responsiveness, we restricted our analysis to Caucasian women. Serum was aliquoted upon collection and stored at -80°C. All participants subsequently received treatment in accordance with a standardised triple DMARD therapeutic regimen, as previously described[12]. Patients were evaluated after 6 months of therapy and classified as EULAR responders (i.e. good or moderate) or non-responders[13]. The study was approved by Melbourne Health Research and Ethics Committee (2010.293).

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. Reads were aligned to the human genome hg19, then mapped to annotations within the Database of small human noncoding RNAs (DASHR)[14]. Any RNA which did not achieve a count per million of mapped reads greater than 1 in at least 12 samples

was filtered out from the data, leaving 1487 sncRNAs for analysis. Hierarchical clustering and principle component analysis for all 1487 snRNAs analysed are included in Supplementary Figure 1. Differential expression analysis was performed using the edgeR and limma software packages. Correlations between RNA expression and DAS28-ESR values were calculated using edgeR's likelihood ratio test. P-values were adjusted using the Benjamini and Hochberg method. Read counts, normalisation 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 Methods**.

Results

5 sncRNAs are differentially expressed at baseline between responders and non-responders

To identify sncRNAs 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 sncRNAs were identified with an adjusted P-value (adj. *p*-value) < 0.05 (**Supplementary Table 2**). These consisted of 2 tRNAs, chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC; a 5S rRNA, 5S-L612; a 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 non-responders (**Figure 1b-c**). Analysis details for all 1487 detected sncRNAs are specified in **Supplementary Table 2**.

5' tRNA halves are enriched in non-responders to triple DMARD therapy

Because circulating tRNA fragments are elevated in various diseases[15], we investigated whether sequencing reads attributed to the two differentially expressed tRNAs wholly or partially aligned to the full-length tRNA gene. Strikingly, all reads attributed to

chr1.tRNA131-GlyCCC and chr2.tRNA13-AlaCGC only partially aligned to the full-length gene sequences (**Figure 2a**). Furthermore, the majority of reads were derived from the 5' end of the tRNA gene and were truncated at the anti-codon loop (**Figure 2b**), consistent with 5' translation interfering tRNA halves (tiRNAs)[15].

sncRNA associations with clinical parameters

To explore circulating sncRNAs as potential biomarkers of disease activity, associations between sncRNA expression and clinical variables were investigated (**Supplementary Tables 3-12**). chr1.tRNA131-GlyCCC expression was significantly decreased in patients positive for anti-citrullinated protein antibody (ACPA) (2.26 log2-fold; adj. *p*-value = 0.0497) and rheumatoid factor (RF) (chr1.tRNA131-GlyCCC (2.57 log2-fold; adj. *p*-value = 7.15e-5). A significant positive correlation with tender 28-joint count (TJC28) (r = 0.14, adj. *p*-value = 7.19e-6) was also apparent. piR-35982 was significantly decreased in patients positive for RF 1.36 log2-fold; adj. *p*-value = 0.049) and showed modest, negative correlations with C-reactive protein (CRP) (r = -0.03, adj. *p*-value = 0.03) and erythrocyte sedimentation rate (ESR) (r = -0.06, adj. *p*-value = 0.03). U2-L166 showed a positive correlation with TJC28 (r = 0.20, adj. *p*-value = 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 RA patients. In this study, we identify five circulating sncRNAs which are differentially expressed between responders and non-responders to triple DMARD therapy. If confirmed, these circulating sncRNAs 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' tiRNAs were significantly elevated in non-responders to triple DMARD therapy. tiRNAs are evolutionary conserved RNAs that are produced following cleavage of full-length tRNAs at the anti-codon loop by the RNase, angiogenin[15]. Fragmentation of tRNAs extends tRNA function beyond facilitating protein synthesis[15], with tiRNAs 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 pro-inflammatory cell behaviour in RA.

sncRNAs 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 sncRNAs 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 sncRNAs 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 DMARDs[10]. These miRNAs 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, sncRNAs associated with response to therapy must be distinguished from sncRNAs that are associated with more severe disease. We found expression of chr1.tRNA131-GlyCCC 5' tiRNA was significantly increased in non-responders to triple DMARD therapy, with the lowest *p*-value of all sncRNAs analysed. Consistent with poorer treatment outcomes for patients negative for ACPA and RF[20], levels of chr1.tRNA131-GlyCCC were significantly increased in these patients. Furthermore, a significant positive correlation between chr1.tRNA131-GlyCCC levels and TJC28 scores (r = 0.14, adj. *p*-value = 7.19e-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 parameters suggest these sncRNAs are more likely to reflect DMARD responsiveness.

In summary, we identify five circulating sncRNAs in patients with early RA, that might represent clinically useful biomarkers of subsequent triple DMARD responsiveness. In addition, sncRNAs such as tiRNAs 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 Caucasian women. We also found a significant difference in RF positivity between responders and non-responders in our patient cohort. While RF status is reported to be a poor predictor of methotrexate response[21], its ability to predict the response to triple DMARDs has not been extensively investigated. The sncRNAs 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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Figure 1: sncRNAs differentially expressed between responders and non-responders

a) MA plot of counts per million reads (CPM) vs fold change for all 1487 sncRNA analysed. sncRNAs with an adjusted P-value < 0.05 are indicated in red and labelled. b) Supervised heatmap showing relative expression of significantly differentially expressed sncRNAs 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 *inter-quartile range. Mean values are indicated.

Figure 2: tRNA reads are truncated at the anti-codon 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 coloured accordingly. b) Position of sequence reads in relation to tRNA secondary structures. For both tRNA, reads are attenuated at the anti-codon-loop. Nucleotides within sequencing reads are indicated in black; absent nucleotides are indicated in grey. Nucleotide variations from canonical gene sequence are specified with an *.

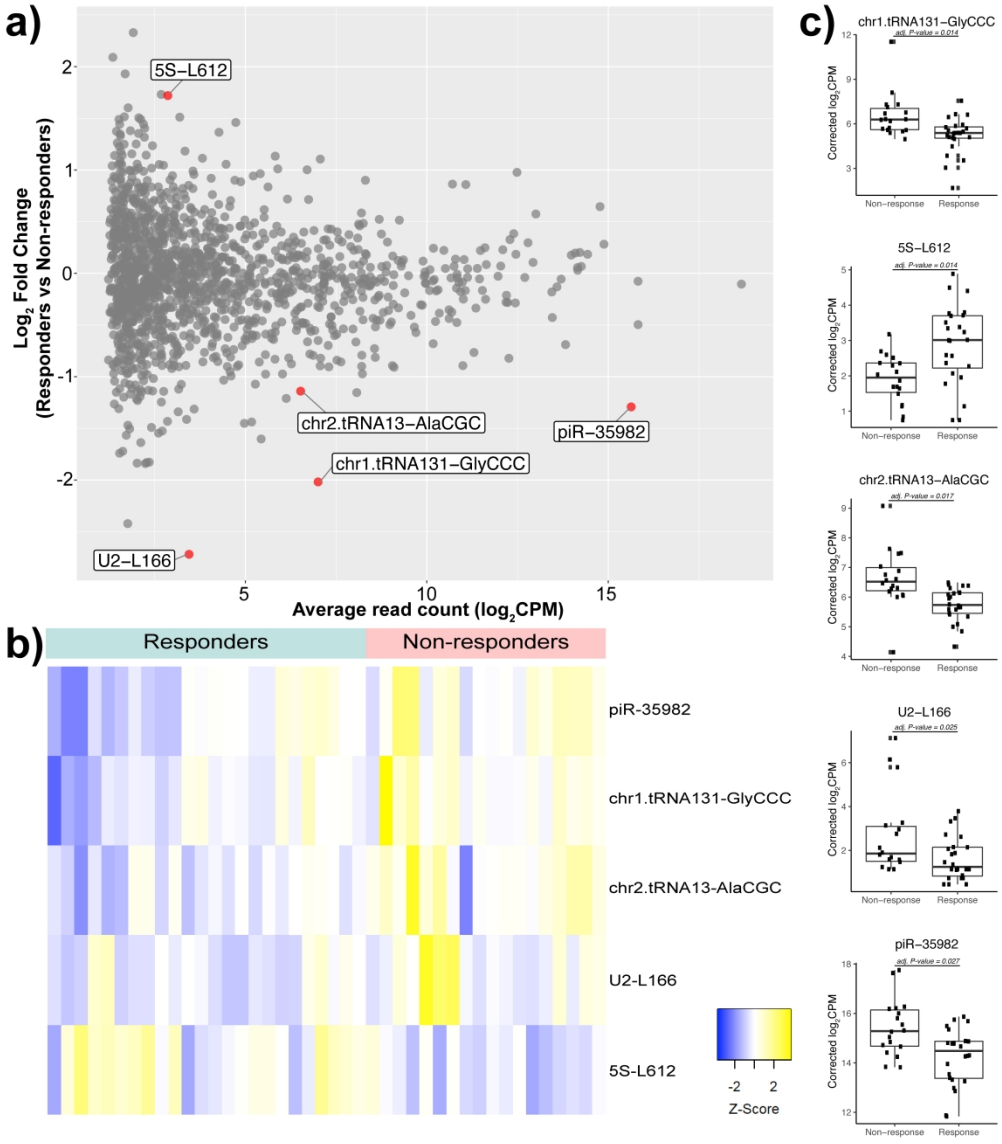

Figure 1

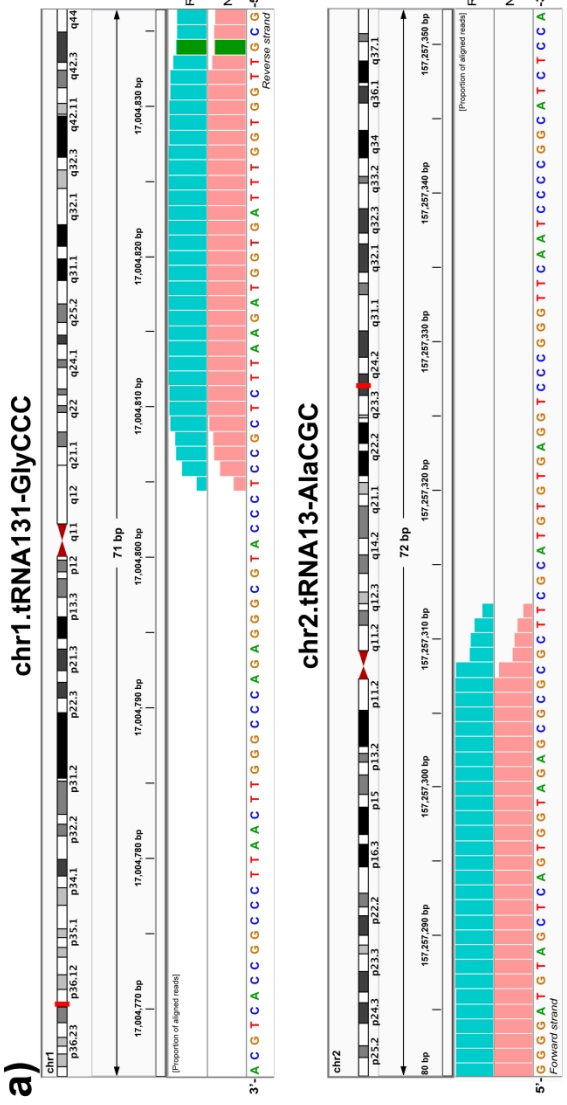

Figure 2A

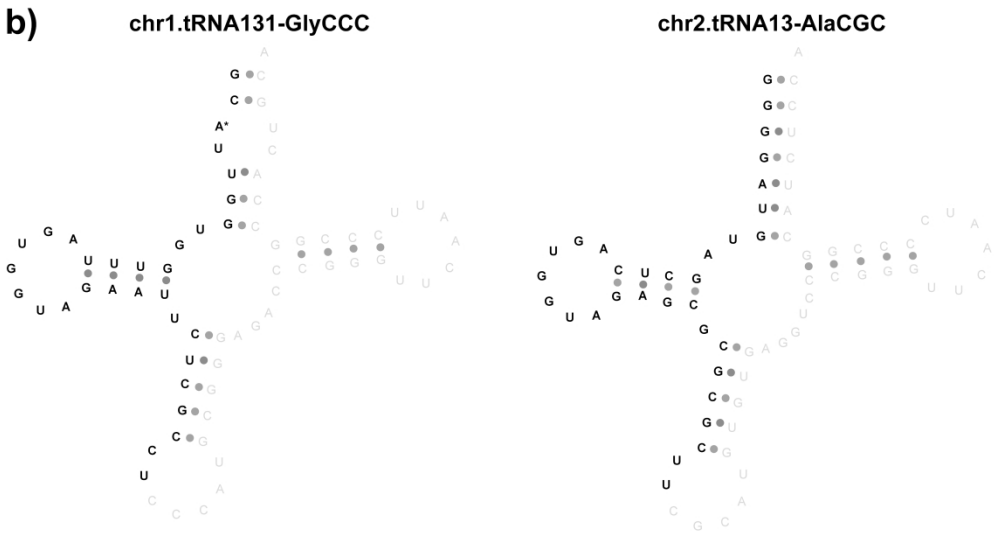

Figure 2B

Table 1: Patient demographics and baseline clinical parameters

	Responders	***Non-responders***	***P-value***
N	24	18	1
% female	100	100	1
Age – mean (s.d.)	60.4 (14.5)	54.6 (11.5)	0.782
Weeks of polyarthritis – mean (s.d.)	23.9 (19.0)	40.2 (69.5)	0.432
Anti-citrullinated protein antibody (% positive)	75	52.9	0.189
Rheumatoid factor (% positive)	91.7	44.4	*0.001*
DAS28-ESR – mean (s.d.)	5.7 (1.1)	5.6 (1.3)	0.997
Swollen 28-joint count – mean (s.d.)	9.7 (5.7)	10.0 (7.5)	0.989
Tender 28-joint count – mean (s.d.)	11.9 (6.5)	15.5 (8.6)	0.863
C-reactive protein (mg/l) – mean (s.d.)	25.5 (22.8)	13.3 (21.1)	0.557
Erythrocyte sedimentation rate (mm/h) – mean (s.d.)	39.5 (30.7)	25.3 (15.0)	0.496
White cell count (1 X 10^6 cells/ml) – mean (s.d.)	8.6 (2.6)	7.4 (3.8)	0.956

Anti-citrullinated protein antibody and rheumatoid factor positivity analysed with Fisher's exact test. All other parameters analysed with Student's t test.