WEHI
brighter together

This is the author's peer reviewed manuscript version of a work accepted for publication.

Publication details:	Foers AD, Garnham AL, Smyth GK, Proudman SM, Cheng L, Hill AF, Pang KC, Wicks IP. Circulating small non-coding RNA biomarkers of response to triple DMARD therapy in Caucasian women with early rheumatoid arthritis. *Journal of Rheumatology.* 2020 47(12):1746-1751
Published version Is available at:	https://doi.org/10.3899/jrheum.191012

Changes introduced as a result of publishing processes such as copy-editing and formatting may not be reflected in this manuscript.

The Journal of Rheumatology

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

Andrew D. Foers, Alexandra L. Garnham, Gordon K. Smyth, Susanna M. Proudman, Lesley Cheng, Andrew F. Hill, Ken C. Pang and Ian P. Wicks

1. Sign up for TOCs and other alerts
   http://www.jrheum.org/alerts

2. Information on Subscriptions
   http://jrheum.com/faq

3. Information on permissions/orders of reprints
   http://jrheum.com/reprints_permissions

The Journal of Rheumatology is a monthly international serial edited by Earl D. Silverman featuring research articles on clinical subjects from scientists working in rheumatology and related fields.

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

Andrew D. Foers[1], Alexandra L. Garnham[1], Gordon K. Smyth[2], Susanna M. Proudman[3], Lesley Cheng[4], Andrew F. Hill[4], Ken C. Pang[5*], Ian P. Wicks[6*]

[1] The Walter and Eliza Hall Institute of Medical Research, and Department of Medical Biology, University of Melbourne, Parkville, Australia

[2] The Walter and Eliza Hall Institute of Medical Research, and School of Mathematics & Statistics, University of Melbourne, Parkville, Australia

[3] Rheumatology Unit, Royal Adelaide Hospital, and Discipline of Medicine, University of Adelaide, Adelaide, Australia

[4] Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Australia

[5] Departments of Paediatrics, Psychiatry and Adolescent Medicine, University of Melbourne, and Murdoch Children's Research Institute, Parkville, Australia

[6] The Walter and Eliza Hall Institute of Medical Research, and Department of Rheumatology, Royal Melbourne Hospital, Parkville, Australia

* KCP and IPW contributed equally.

Correspondence:

Ian P. Wicks
wicks@wehi.edu.au
The Walter & Eliza Hall Institute
Parkville, VIC, AUS, 3052
Phone: +61 3 9345 2466
Fax: +61 3 9347 0852

Ken C. Pang
ken.pang@mcri.edu.au
Murdoch Children's Research Institute,
Parkville, VIC, AUS, 3052
Phone: +61 9345 5986
Fax: +61 3 9348 1391

Academic degrees:

Andrew D. Foers, PhD; Alexandra L. Garnham, PhD; Gordon K. Smyth, PhD
Susanna M. Proudman, MB BS; Lesley Cheng, PhD; Andrew F. Hill, PhD; Ken C. Pang, MB BS, PhD; Ian P. Wicks, MB BS, PhD

Conflicts of interest:

SMP reports grants from Actelion Pharmaceuticals and Melrose, outside the submitted work.

Key indexing terms:

Rheumatoid arthritis; small non-coding RNA; biomarker; DMARDs; translation interfering tRNA halves.

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

3

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

5

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.

Acknowledgements

Author contributions: All authors listed contributed and fulfil authorship criteria.

Ethics approval: The study was approved by the Royal Adelaide Hospital and The Walter and Eliza Hall Institute human research ethics committees.

References

1. Fritz JV, Heintz-Buschart A, Ghosal A, Wampach L, Etheridge A, Galas D, et al. Sources and functions of extracellular small rnas in human circulation. Annu Rev Nutr 2016;36:301-36.
2. Anfossi S, Babayan A, Pantel K, Calin GA. Clinical utility of circulating non-coding rnas — an update. Nat Rev Clin Oncol 2018;15:541-63.
3. O'Dell JR, Haire CE, Erikson N, Drymalski W, Palmer W, Eckhoff PJ, et al. Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three medications. N Engl J Med 1996;334:1287-91.
4. Proudman SM, Keen HI, Stamp LK, Lee ATY, Goldblatt F, Ayres OC, et al. Response-driven combination therapy with conventional disease-modifying antirheumatic drugs can achieve high

response rates in early rheumatoid arthritis with minimal glucocorticoid and nonsteroidal anti-inflammatory drug use. Semin Arthritis Rheum 2007;37:99-111.

5. Rantalaiho V, Korpela M, Hannonen P, Kautiainen H, Jarvenpaa S, Leirisalo-Repo M, et al. The good initial response to therapy with a combination of traditional disease-modifying antirheumatic drugs is sustained over time: The eleven-year results of the finnish rheumatoid arthritis combination therapy trial. Arthritis Rheum 2009;60:1222-31.

6. Duroux-Richard I, Pers Y-M, Fabre S, Ammari M, Baeten D, Cartron G, et al. Circulating mirna-125b is a potential biomarker predicting response to rituximab in rheumatoid arthritis. Mediators Inflamm 2014;2014:342524.

7. Piao X, Hu J. Circulating microrna-125b expression predicts clinical response in patients with rheumatoid arthritis treated by tumor necrosis factor inhibitors. Int J Clin Exp Pathol 2017;10:1809-16.

8. Cuppen BVJ, Rossato M, Fritsch-Stork RDE, Concepcion AN, Schenk Y, Bijlsma JWJ, et al. Can baseline serum micrornas predict response to tnf-alpha inhibitors in rheumatoid arthritis? Arthritis Res Ther 2016;18:189.

9. Sode J, Krintel SB, Carlsen AL, Hetland ML, Johansen JS, Hørslev-Petersen K, et al. Plasma microrna profiles in patients with early rheumatoid arthritis responding to adalimumab plus methotrexate vs methotrexate alone: A placebo-controlled clinical trial. J Rheumatol 2018;45:53.

10. Filková M, Aradi B, Šenolt L, Ospelt C, Vettori S, Mann H, et al. Association of circulating mir-223 and mir-16 with disease activity in patients with early rheumatoid arthritis. Ann Rheum Dis 2014;73:1898–904.

11. Castro-Villegas C, Perez-Sanchez C, Escudero A, Filipescu I, Verdu M, Ruiz-Limon P, et al. Circulating mirnas as potential biomarkers of therapy effectiveness in rheumatoid arthritis patients treated with anti-tnfalpha. Arthritis Res Ther 2015;17:49.

12. Proudman SM, Cleland LG, Metcalf RG, Sullivan TR, Spargo LD, James MJ. Plasma n-3 fatty acids and clinical outcomes in recent-onset rheumatoid arthritis. Br J Nutr 2015;114:885-90.

13. van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the european league against rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary american college of rheumatology and the world health organization/international league against rheumatism criteria. Arthritis Rheum 1996;39:34-40.

14. Leung YY, Kuksa PP, Amlie-Wolf A, Valladares O, Ungar LH, Kannan S, et al. Dashr: Database of small human noncoding rnas. Nucleic Acids Res 2016;44:D216-D22.

15. Schimmel P. The emerging complexity of the trna world: Mammalian trnas beyond protein synthesis. Nat Rev Mol Cell Biol 2017;19:45.

16. Li S, Shi X, Chen M, Xu N, Sun D, Bai R, et al. Angiogenin promotes colorectal cancer metastasis via tirna production. Int J Cancer 2019;0.

17. Murata K, Yoshitomi H, Tanida S, Ishikawa M, Nishitani K, Ito H, et al. Plasma and synovial fluid micrornas as potential biomarkers of rheumatoid arthritis and osteoarthritis. Arthritis Res Ther 2010;12:R86.

18. Murata K, Furu M, Yoshitomi H, Ishikawa M, Shibuya H, Hashimoto M, et al. Comprehensive microrna analysis identifies mir-24 and mir-125a-5p as plasma biomarkers for rheumatoid arthritis. PLoS One 2013;8:e69118.

19. Aletaha D, Funovits J, Keystone EC, Smolen JS. Disease activity early in the course of treatment predicts response to therapy after one year in rheumatoid arthritis patients. Arthritis Rheum 2007;56:3226-35.

20. Pope JE, Movahedi M, Rampakakis E, Cesta A, Sampalis JS, Keystone E, et al. Acpa and rf as predictors of sustained clinical remission in patients with rheumatoid arthritis: Data from the ontario best practices research initiative (obri). RMD Open 2018;4:e000738.

21. Yu MB, Firek A, Langridge WHR. Predicting methotrexate resistance in rheumatoid arthritis patients. Inflammopharmacology 2018;26:699-708.

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 *.
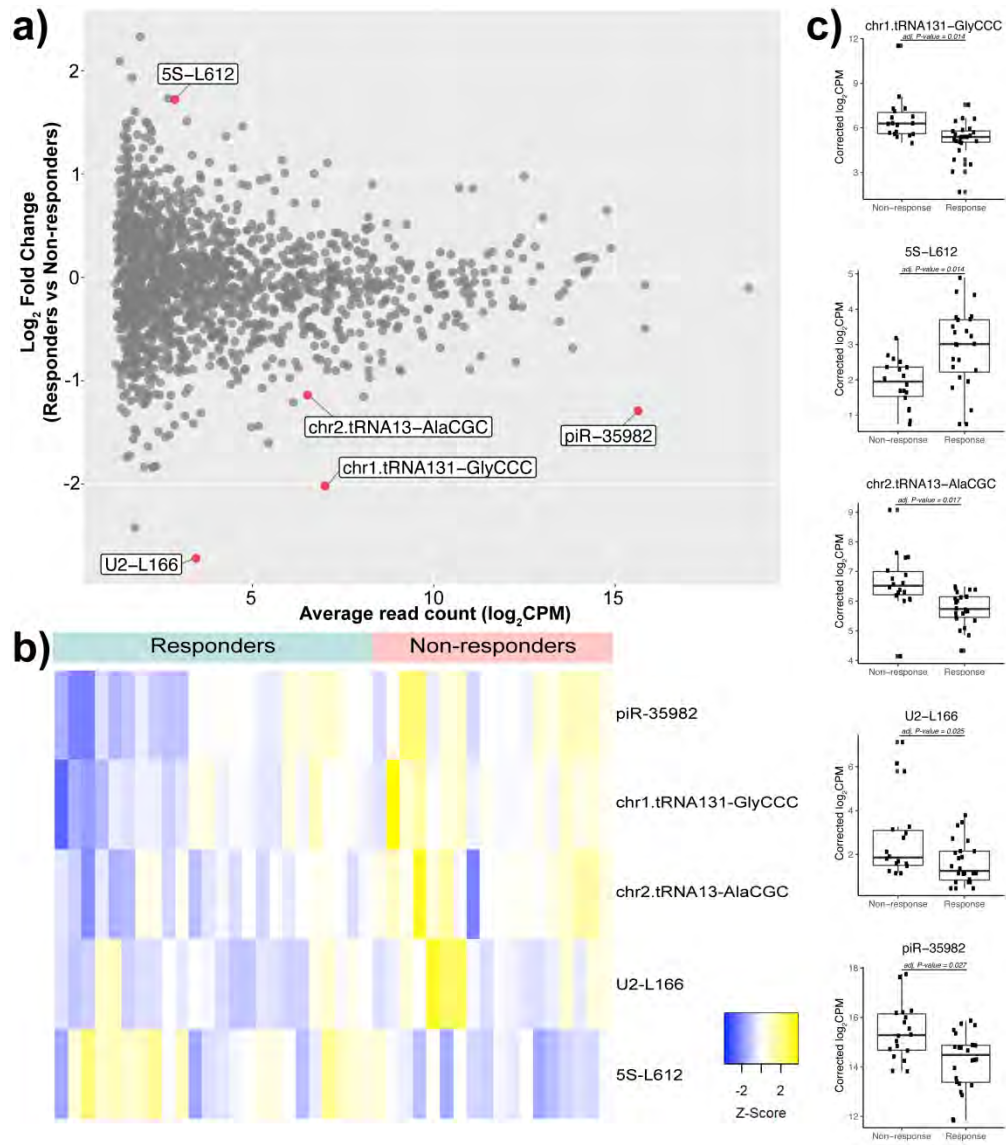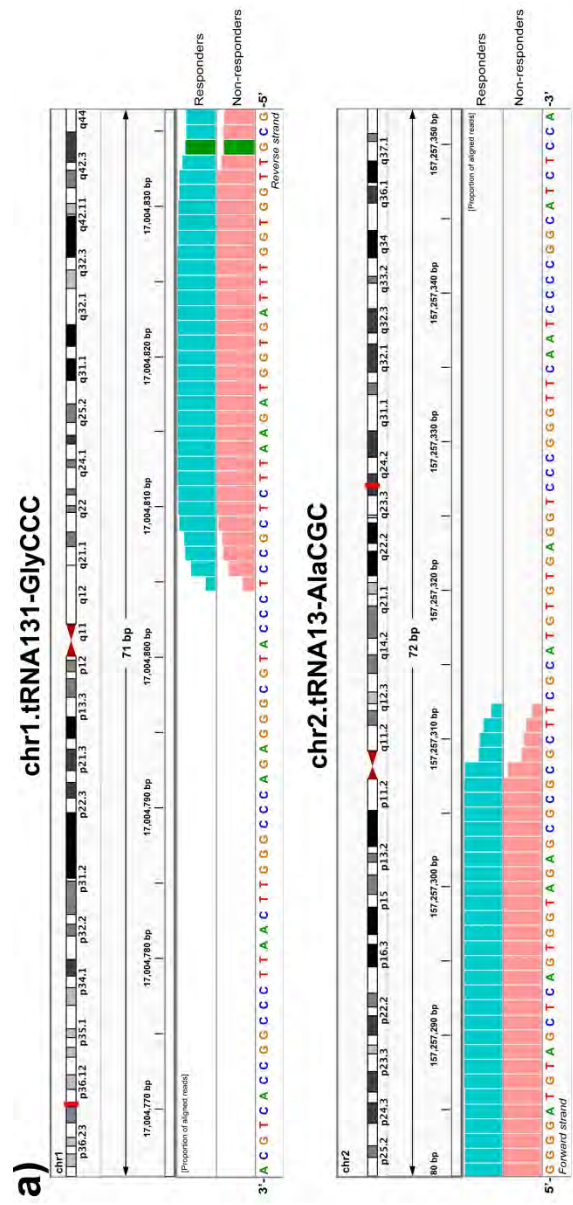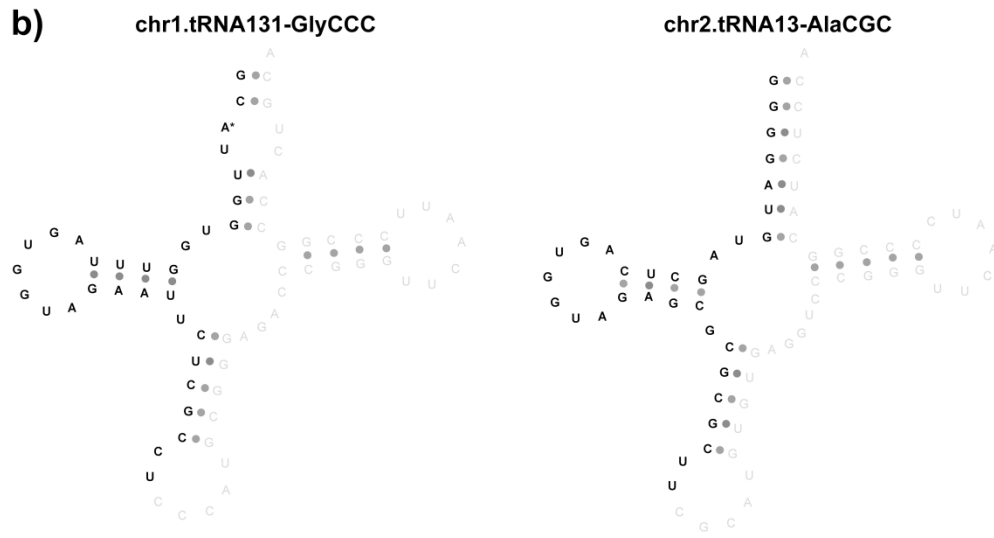
1

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.