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Original article

Study of miRNA interactome in active rheumatoid arthritis patients reveals key pathogenic roles of dysbiosis in the infection–immune network

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Abstract

Objectives. To characterize serum microRNA (miR) and the miR interactome of active RA patients in RA aetiology and pathogenesis.

Methods. The differentially expressed miRs (DEmiRs) in serum of naïve active RA patients (NARAPs, $n = 9$, into three pools) *vs* healthy controls (HCs, $n = 15$, into five pools) were identified with Agilent human miR microarray analysis. Candidate driver genes in epigenetic and pathogenic signalling pathway modules for RA were analysed using miRTarBase and a molecular complex detection algorithm. The interactome of these DEmiRs in RA pathogenesis were further characterized with gene ontology and Kyoto Encyclopaedia of Genes and Genomes.

Results. Three upregulated DEmiRs (hsa-miR-187-5p, -4532, -4516) and eight downregulated DEmiRs (hsa-miR-125a-3p, -575, -191-3p, -6865-3p, -197-3p, -6886-3p, -1237-3p, -4436b-5p) were identified in NARAPs. Interactomic analysis from heterogeneous experimentally validated sources yielded 1719 miR–target interactions containing 5.67% strong and 94.33% less strong experimental evidence. Gene ontology and Kyoto Encyclopaedia of Genes and Genomes analyses allocated the upregulated DEmiRs in the infection modules and the downregulated DEmiRs in the immune signalling pathways. Specifically, these DEmiRs revealed the significant contributions of the intestinal microbiome dysbiosis in the infection–inflammation–immune network for activation of T cells, immune pathways of IL-17, Toll-like receptor, TNF, Janus kinase-signal transducer and activator of transcription, osteoclast cell differentiation pathway and IgA production to the active RA pathogenesis.

Conclusions. Our experiment-based interactomic study of DEmiRs in serum of NARAPs revealed novel clinically relevant miRs interactomes in the infection–inflammation–immune network of RA. These results provide valuable resources for understanding the integrated function of the miR network in RA pathogenesis and the application of circulating miRs as biomarkers for early aetiologic RA diagnosis.

Key words: rheumatoid arthritis, circulating microRNA, interactome, intestinal microbiome, infection-immune network, bioinformatics, biomarkers, interactome

BASIC SCIENCE

Rheumatology key messages

- The reliable specific pathogenic biomarkers for early diagnosis of active RA remain missing.
- We used a new patient-experiment-based approach to characterize the microRNA interactomes in naïve active RA patients.
- This study uncovered the intestinal dysbiosis–infection–inflammation–immune network as a key pathogen of the active RA.

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Introduction

RA is a systemic inflammatory autoimmune disease with unknown pathogenic triggers that persistently activate the inflammation and immune responses [1]. Although RA is characterized primarily by irreversible destruction of affected joints, its clinical manifestations are heterogeneous and complex, with wide individual differences that may involve other organ systems with severe complications and comorbidities [2]. There is currently no cure for RA. The early use of DMARDs may inhibit the underlying immune process and avoid long-term damage to achieve tight disease control and reach a low disease activity state or full suppression of the disease activity, i.e. clinical remission [3]. The therapeutic window for clinical remission of active RA, however, is usually very narrow. Most RA patients have missed the window of early diagnosis and treatment and progressed to chronic phases that result in handicap, disability, social dysfunction and premature death. Early aetiologic and pathogenic diagnosis, therefore, is the key for effective treatment and most desirable outcomes of active RA. Unfortunately, it remains impossible to make early pathogenic diagnosis of active RA due to the lack of knowledge about the exact causes and the complex pathological mechanisms of the deterioration of RA progress with persistent and systemic autoimmune and inflammation [4–6]. To date, therefore, no reliable specific biomarkers are available for early pathogenic diagnosis of RA.

Despite accumulated evidence for the involvement of genetic and environmental factors, including some specific bacterial and viral infectious agents, and disturbances of intestinal, lung and oral microbiota in RA development and evolution, there are no clear and reliable predisposing factors in most RA patients [4–6]. As crucial epigenetic factors bridging the environmental triggering agents to genes, the noncoding microRNAs (miRs) destabilize mRNA and/or inhibit protein translation to affect the final RA epigenome state. The complex rheumatic responses to pathogenic triggers with systemic inflammatory autoimmune require orchestration of gene regulatory networks that are modulated, in part, by miRs. Recent studies have accumulated some compelling evidence for the crucial roles of endogenous miRs in regulating and controlling both innate and adaptive immunity, which strongly suggested that miRs may represent an additional class of genes involved in the development of autoimmune disorders [3, 7–10]. In response to the influence of environmental factors, the miRs associate with Argonaute proteins to direct post-transcriptional gene suppression via base-pairing with target transcripts, and/or interact with the RNA-induced silencing complex to regulate a wide variety of other genes by either preventing the translation or inducing the cleavage of complementary mRNAs [7]. As crucial epigenetic factors that bridge the environmental triggering agents to genes, miRs destabilize mRNA and/or inhibit protein translation to affect the final RA epigenome state. Deregulation of miRs can cause autoimmune diseases, including RA [3, 7–10]. It has been reported that the expression of miR-124a was significantly decreased in RA synovial fibroblasts or peripheral blood monocytes, while the expression of miR-15, -16, -132, 146a, -155 and -223 was increased [11]. Another study, however, found reduced levels of miR-223 and miR-16 in the sera of patients with early RA compared with those with established RA, speculating that these miRs may be taken up in early stage of RA by cells in inflammatory responses [12]. Low levels of miR-124a could recruit mononuclear phagocytes into the joints via MCP-1, thereby fuelling inflammation in RA [13]. For an overview of the advances in the study of miRs as biomarkers for diagnosis, prognosis and response to treatment in different pathologies of RA, please refer to a recently published excellent review article by Moran-Moguel *et al*. [14].

Theoretically, therefore, serum miRs may be valuable biomarkers for early diagnosis, disease monitoring and evaluation of response to treatment in RA [10]. However, in the nature of the complex rheumatic responses to pathogenic triggers with systemic inflammatory autoimmune, an orchestrated network of multi-miRs and their interacting partners. Identifying and characterizing such miRs interactomes is very difficult. On one hand, the imperfect Watson–Crick pairing of a single-stranded miR allows a single miR to interact with hundreds of mRNA targets. On the other hand, very little is currently known about the expression profiles and the corresponding functions of differentially expressed miRs (DEmiRs) in the serum of active RA patients. Therefore, it is of paramount importance to elucidate the regulation of the complex pathogenesis and the mechanistic characteristics of multisystem invasions of RA at the entire disease-associated genetic and epigenetic levels by the miR interactome network in naïve active RA patients (NARAPs), who have never been treated with any DMARDs, glucocorticoids or other immunosuppressive drugs [4].

In this study, we first identified the DEmiRs in the serum of NARAPs and then used experiment-based bioinformatics to analyse candidate driver genes in signalling pathway modules. We further applied gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) to analyse the functional roles of these DEmiRs in pathogenesis of RA. Notably, our initial exploration of these data has revealed numerous clinically relevant interactions involving miRs and target genes previously implicated in RA. Also, the miR interactome identified in this study points to a coordination of miR activities in dysbiosis of the gut microbiome as the key to the orchestrated significant contributions of the intestinal infection–immune network for production of IgA and activation of T (Th1, Th2, Th17) cells, and several other common immune signalling pathways of IL-17, Toll-like receptor, TNF and Janus kinase-signal transducer and activator of transcription (JAK-STAT), as well as the osteoclast cell differentiation pathway, to the active RA

immunopathogenesis. In addition, signalling of infection, especially the virus and Toxoplasmosis infections, was shown to be related to RA pathogenesis. Overall, this work not only represents an initial step towards characterizing the diverse landscape of miR–target interactions (MTIs) across normal and diseased human RA, but also offers clues that may facilitate the translation of genetic studies of complex RA into novel or refined pathogenic diagnosis and therapeutics of active RA patients.

Methods

Ethics statement

The procedures described in this study were approved by Ethics Committee of the People's Hospital of Ningxia Hui Autonomous Region, China. All volunteer participants signed a written informed consent. The study was carried out in accordance with the Helsinki Declaration.

This research was carried out with involvement of 9 NARAPs and 15 healthy controls (HCs). For more details of the inclusion and exclusion criteria please see supplementary Table S1, available at *Rheumatology* online. While they consented to participate in the research with physical and laboratory examinations and donate blood samples, the patients and HCs were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Patients and HCs were not invited to contribute to the writing or editing of this document for readability or accuracy.

More detailed methods are described in online supplementary File S1, available at *Rheumatology* online. Briefly, Agilent human miR microarray (Agilent Technologies, Release 21.0, 8*60K, containing 2570 miRs, Design ID: 070156) was used to identify the differentially expressed miRs (DEmiRs) in serum of NARAPs and HCs; miRTarBase and molecular complex detection algorithm were used to analyse the candidate driver genes and interactomes for RA in epigenetic and pathogenic signalling pathway modules. GO and KEGG were used to further characterize the functions of these DEmiRs in RA pathogenesis.

Results

DEmiRs in serum of NARAPs

Under the inclusion criteria of $\log_2$ (fold change) >1 and $P<0.05$ (supplementary Table S2, available at *Rheumatology* online), the Agilent human miR microarray was performed. Out of 2570 miRs, 11 significant DEmiRs were identified at either higher (hsa-miR-187-5p, -4516, -4532) or lower (hsa-miR-125a-3p, -575, -191-3p, -6865-3p, -197-3p, -6886-3p, -1237-3p, -4436b-5p) levels in NARAPs serum compared with HCs (Fig. 1). All the DEmiRs had a false discovery rate <0.05 (supplementary Table S2 and Figs S1 and S2, available at *Rheumatology* online). These inter-correlated quantitative dependent variables were further evaluated and confirmed by principal component analysis (supplmentary Fig. S3, available at *Rheumatology* online).

Experimentally validated MTIs

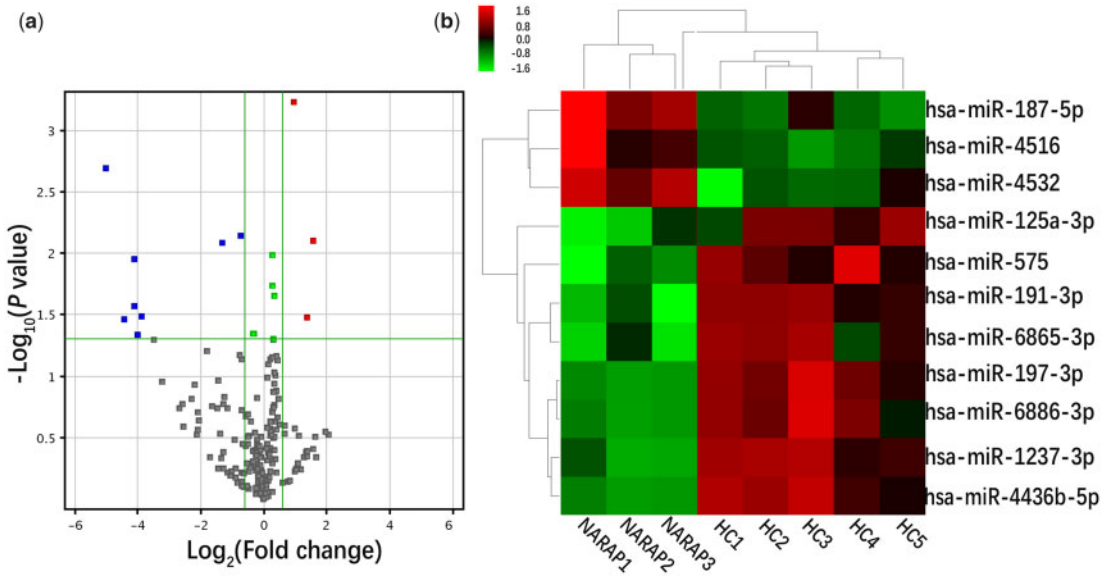
To establish MTIs, and further elucidate and characterize the functions of a given miR, experimental verification was performed using the updated miRTarBase 7.0 database, which provides more comprehensive collection of experimentally supported MTIs and a higher accuracy rate of target prediction [15]. Of the 1719 experimentally determined MTIs, 5.67% contained strong experimental evidences (quantitative RT-PCR, 1.37%; ELISA, 1.05%; immunohistochemistry, 1.23%; western blot, 1.01%; luciferase reporter assay, 1.01%), and 94.33% contained less strong experimental evidences (microarray, 3.15%; Crosslinking-immunoprecipitation and high-throughput sequencing (CLIP-seq) datasets, 85.7%; Crosslinking-ligation and sequencing of hybrids (CLASH), 5.48%) (Fig. 2a). as shown in Fig. 2b and c, CLIP (including HITS-CLIP and PAR-CLIP) [16] and CLASH [17] incorporated high-throughput sequencing could also be widely used to validate miR targets. MTI networks of each DEmiR (except hsa-miR-6865-3p and hsa-miR-6886-3p) were constructed using Cytoscape. We identified 1606 non-repetitive potential target genes, including 1184 genes (1284 MTIs) for the six downregulated miRs (Fig. 2b) and 422 genes (435 MTIs) for the three upregulated miRs in active RA (Fig. 2c). In the MTIs network, certain target genes were regulated by more than one miR.

GO term and KEGG pathway enrichment analysis of DEmiRs

Top 10 (adjusted and ranked by $P<0.05$) dysregulated GO processes [including Molecular Function (MF), Cellular Component (CC) and Biological Process (BP)] were analysed according to the experimentally validated MITs of DEmiRs using the DAVID functional annotation tool [18]. Fig. 3a shows the GO term enrichment of the downregulated DEmiRs, characterized with (i) the CC distributions of intracellular organelle/nucleus/cytoplasm; (ii) MF of nucleic acids (DNA, RNA, miR)/ATP/enzyme/metal ion and cation bindings; and (iii) BP of cellular metabolic/biosynthetic regulation. Fig. 3b shows the main GO term enrichment of the upregulated DEmiRs, characterized with (i) major CC distributions in the complexes of RNA-induced silencing complex/RNA interference effector/nuclear and RNA polymerase II transcription factors, and nuclear pore; (ii) MF of protein kinase inhibitor/regulator activity; and (iii) BP of negative regulation of protein modification/phosphorylation/transferase activity.

The KEGG pathway analysis of the downregulated DEmiRs (Fig. 4a), adjusted and ranked by $P<0.05$, clustered mainly in the immune pathways (intestinal immune network for IgA production and nuclear factor-κB signalling pathway) or immune-related diseases (such as RA).

Fig. 1 Identification of differentially expressed miRs in NARAPs and HCs



(**a**) Volcano plot of DEmiRs. Points above the green line indicate miRs with statistically significant ($P < 0.05$). The *x*-axis shows the $\log_2$ (fold change) and the *y*-axis shows the $-\log_{10}$ (*P*-value) for each miR. The red points in the plot represent the up-regulated miRs (fold change ≥ 2.0, $P < 0.05$), while the blue ones represent the down-regulated miRs (fold change ≤ -2.0, $P < 0.05$). (**b**) Two-way hierarchical cluster analysis of 11 DEmiRs. The *x*-axis denotes samples and the *y*-axis denotes miR. The colour scale indicates that the gene expression varies from relatively low (green) to high (red). miR: microRNA; DEmiRs: differentially expressed miRs; NARAPs: naïve active RA patients ($n = 9$, pooled into three pools); HCs: healthy controls ($n = 15$, pooled into five pools).

In addition, these DEmiRs were also clustered in the infection-related disease pathways (such as vibrio cholerae infection, legionellosis, HTLV-1 infection, measles and influenza A), and bladder cancer. While adjusted and ranked by % terms per group (Fig. 4b) and by % genes per group (Fig. 4c), the key pathway groups were enriched in signalling pathways of Ca^{2+}, ErbB, mammalian target of rapamycin, phospholipase D, IL-17, FcγRI and RA.

The KEGG pathway analysis of the upregulated eight DEmiRs clustered (adjusted and ranked by $P < 0.05$) mainly in the infection disease category, especially virus infection diseases and in multiple immune pathways, as well as cancers (Fig. 4d). While adjusted and ranked by % terms per group (Fig. 4e) and by % genes per group (Fig. 4f), cell cycle, EBV infection, small cell lung cancer, FoxO signalling pathway, advanced glycation endproducts-receptor for advanced glycation endproducts signalling pathway in diabetic complications, and pancreatic cancer were enriched.

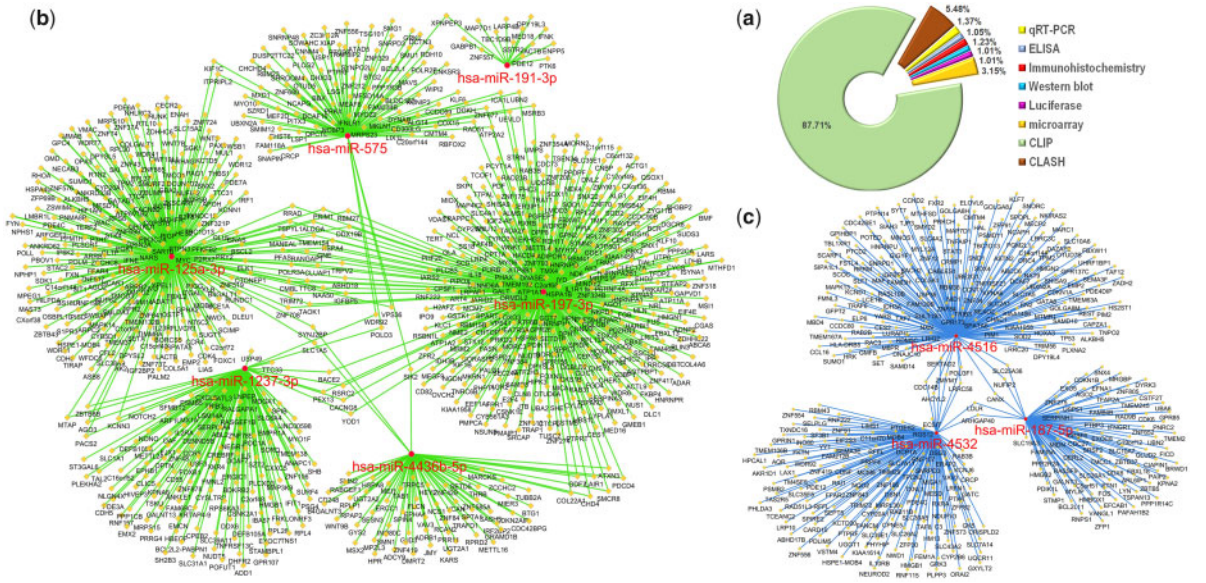
The RA-associated miR-based protein–protein interaction networks

Fig. 5a shows the downregulated DEmiR-based protein–protein interaction (PPI) network, which was based on the information of experimentally verified target genes and visualized by Cytoscape. The PPI network was consisted of 223 GO terms and 299 GO term connections (after merging) among 1184 validated target genes with 100% recognized by ClueGo (a combined score >0.4).

Fig. 6a shows the upregulated DEmiR-based signalling pathway network that consisted of 57 GO terms and 70 GO term connections (after merging) among 422 validated target genes with 100% recognized by ClueGo (a combined score >0.4) to show the interactions with each other. The infection pathways were involved in the immunopathogenesis of RA via some key immune pathways (including p53, JAK-STAT, Wnt, Th17 cell differentiation, Th1 and Th2 cell differentiation signalling pathway).

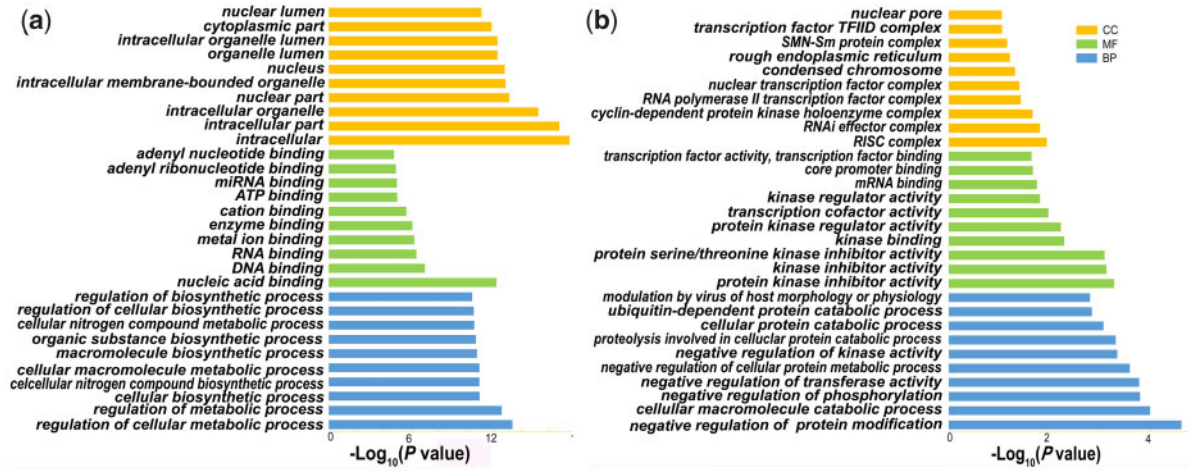
Protein complexes in these collected PPI data sets were automatically predicted using MCODE. Algorithms for finding clusters or locally dense regions based on network flow/minimum cut theory or spectral clustering [19, 20] matched 16 modules to the conditions of the downregulated miRs using Cytoscape MCODE plugin (Fig. 5b). Cluster 2 (nodes 23, edges 84) denoted that the key pathway of intestinal immune network for IgA production interacted with RA and several immune diseases (such as Type 1 diabetes mellitus, IBD, autoimmune thyroid disease) by miR-1237-3p-regulated CD28, miR-1237-3p, -197-3p, -125a-3p and -6886-3p-regulated CD28 and HLA-DMB. Cluster 16 (nodes 6, edges 6) indicated that RA might directly interact with

Fig. 2 The experimental validation of MTIs and regulatory networks



(**a**) The experimental validation of MTIs and regulatory network of down- (**b**) or up-regulated (**c**) miRs and their target genes in patients with active RA. The red circle nodes are DEmiRs, the yellow rhombus nodes are target genes of DEmiRs. miRTarBase is available at http://miRTarBase.mbc.nctu.edu.tw. Visualized by cystoscope. The detailed data information corresponding to (a) can be found in supplementary Table S3, available at *Rheumatology* online, and the target genes presented in (b) and (c) can be found in the supplemental excel file titled 'The target genes for each miRNA', available at *Rheumatology* online. miR: microRNA; MTIs: miR–target interactions; DEmiRs: differentially expressed miRs.

Fig. 3 Top 10 dysregulated GO for validated target genes of DEmiRs between NARAPs and HC

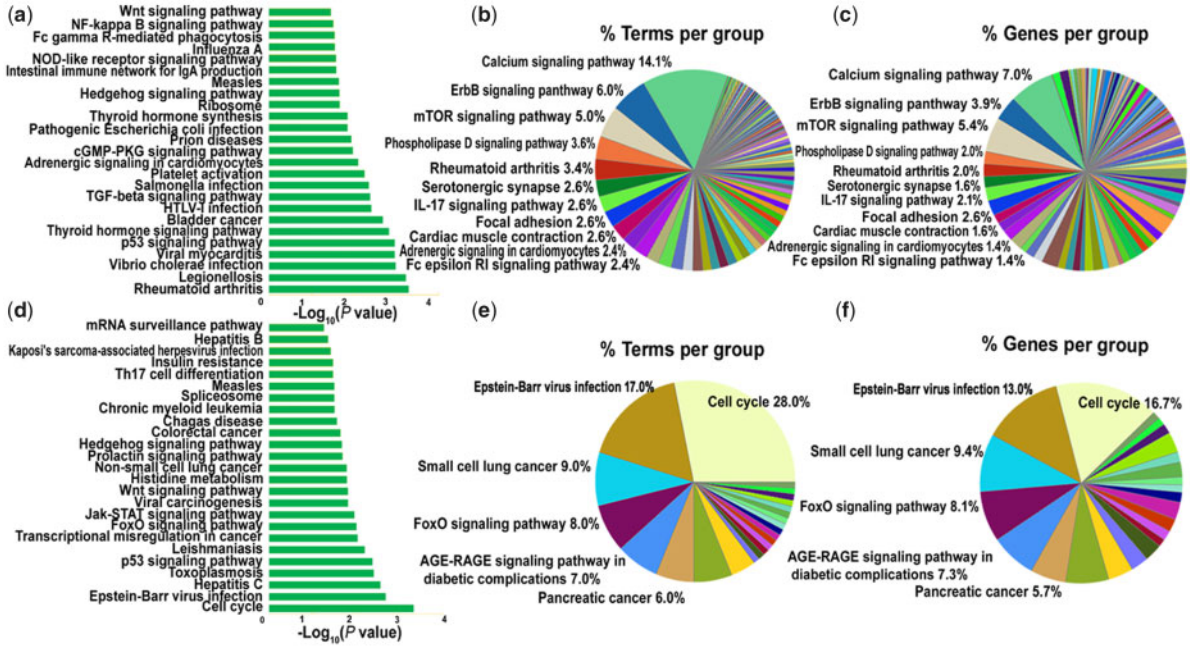


GO terms enrichment analysis of significantly down-regulated miRs (**a**) and up-regulated miRs (**b**) (adjusted and ranked by $P < 0.05$). miR: microRNA; DEmiRs: differentially expressed miRs; NARAPs: naïve active RA patients; HCs: healthy controls; GO: gene ontology; BP: biological process; CC: cellular component, MF: molecular function.

osteoclast differentiation by miR-197-3p and -191-3p-regulated CSF1 and with NK cell mediated cytotoxicity by miR-125a-3p-regulated integrin subunit alpha L. In the PPI network, some key genes, including IL-6 (cluster 9) and CLCL5 (cluster 10), might be regulated by miR-125a-3p and involved in the RA-related KEGG pathway of Toll-like receptor signalling pathway, IL-17 signalling pathway and TNF signalling pathway.

In Fig. 6b, seven pathway modules based on the upregulated miR-related pathway networks were found to show locally dense pathway regions matching with the key protein complexes of the whole networks. The

FIG. 4 KEGG pathway enrichment analysis of significantly dyregulated miRs



All the detailed data information presented in this figure can be found in supplementary Tables S4–7, available at *Rheumatology* online. KEGG: Kyoto Encyclopaedia of Genes and Genomes; miR: microRNA.

miR-4516 regulated targets Mitogen-activated protein kinase 13 and STAT3 were involved in RA-related Toxoplasmosis via Th1, Th2 and Th17 cell differentiation in cluster 3 (nodes 5, edges 8). Leishmaniasis was the key pathway, which may trigger the IL-17 signalling pathway via Fos proto-oncogene, AP-1 transcription factor subunit (FOS) regulated by miR-187-5p in cluster 6 (nodes 3, edges 3).

Discussion

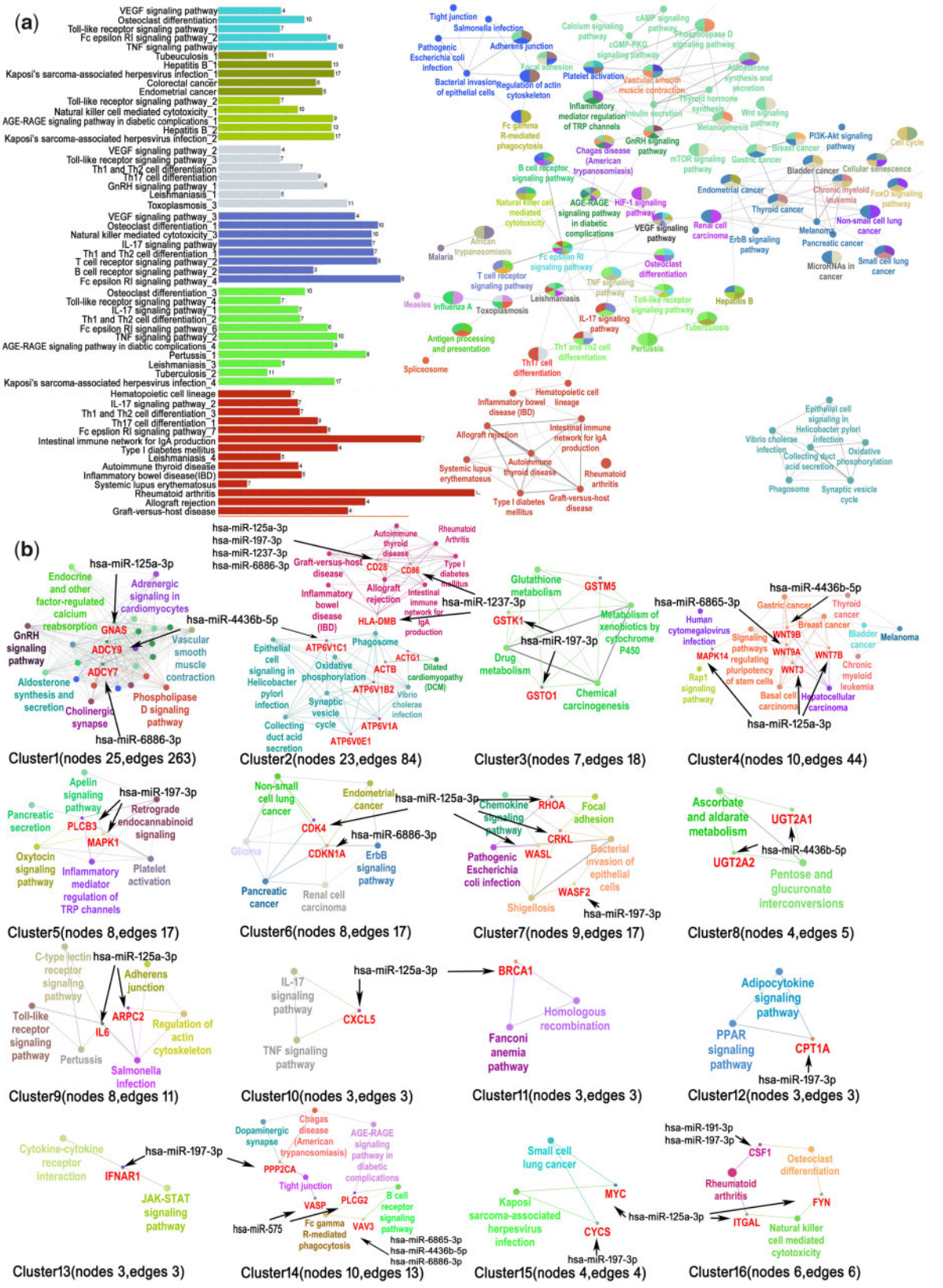
The lack of knowledge on the complex aetiology and pathogenesis of RA has hampered the development of specific biomarkers for early diagnosis and curative therapy for this devastating disease. Although recent studies have provided compelling evidence for a crucial role of several endogenous miRs in regulating and controlling immunity and have been implicated in the pathogenesis of RA, these studies provided very little knowledge about the expression profiles and the corresponding functions of DEmiRs in the serum of NARAPs. Most studies focussed on changes in a single miR in RA patients or animal models. The development of high-throughput detection techniques has greatly enhanced the identification of the RA-related miR expression profile. However, the most common computational approach, which relies on 6- to 8-base-pair perfect seed pairing, is not a generally reliable predictor for an interaction of lsy-6 with a 3′ untranslated region. Thus, experimental verification must be carried out to establish miRs and their target interactions to elucidate the functions of a given miR [15, 21].

In this study, we used a new patient-experiment-based approach to characterize the miR interactomes in untreated active RA patients and made the following major novel findings: (i) three upregulated DEmiRs (hsa-miR-187-5p, -4532, -4516) and eight downregulated DEmiRs (hsa-miR-125a-3p, -575, -191-3p, -6865-3p, -197-3p, -6886-3p, -1237-3p, -4436b-5p) were identified in NARAPs; (ii) 422 targeted genes (435 MTIs) for the three upregulated miRs and 1184 targeted genes (1284 MTIs) for the six downregulated miRs were identified and functionally characterized in the epigenetic and pathogenic signalling pathway modules; and (iii) an intestinal dysbiosis–infection–inflammation–immune network was uncovered as a key pathogen of the active RA. These findings provided novel mechanistic insights into the functional and molecular network of RA pathogenesis and effective experiment-based systematic approaches to the identification of circulating miRs in serum as novel biomarkers for early and pathogenic diagnosis and precision therapy of active RA (supplementary Fig. S4, available at *Rheumatology* online).

The characterization of the down-regulated DEmiRs and miR interactome

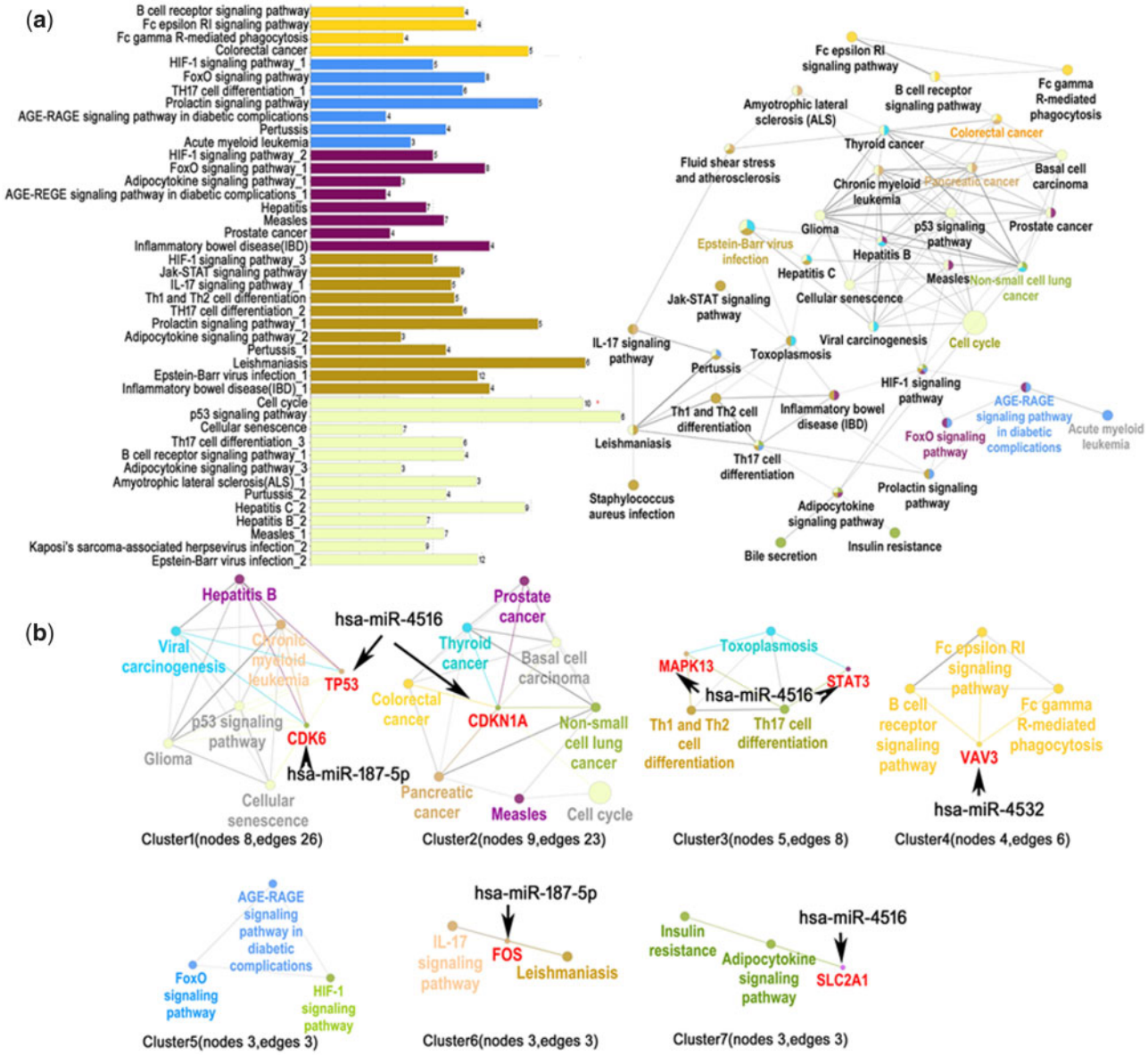
Among the 11 DEmiRs identified in the serum of NARAPs [DAS28-ESR, 4.59 (S.D. 1.73)] there were eight downregulated miRs (hsa-miR-125a-3p, -575, -191-3p, -6865-3p, -197-3p, -6886-3p, -1237-3p, -4436b-5p). Our interactomic analysis of these down-regulated DEmiRs

FIG. 5 The down-expressed miR-based KEGG networks and PPI modules construction



The down-expressed miR-based PPI networks construction (**a**) and modules predicted by the PPI network using the Cytoscape MCODE plugin (**b**) in RA. miR: microRNA; KEGG: Kyoto Encyclopaedia of Genes and Genomes; PPI: protein–protein interaction.

FIG. 6 The up-expressed miR-based KEGG networks and PPI modules construction



The up-expressed miR-based KEGG pathway networks construction (**a**) and modules identified by the PPI network using the Cytoscape MCODE plugin (**b**) in RA. Vertices are KEGG pathways or molecules. Edges are molecular interactions. miR: microRNA; KEGG: Kyoto Encyclopaedia of Genes and Genomes; PPI: protein–protein interaction.

revealed the significant contributions of the orchestrated intestinal biome infection–immune network for production of IgA and activation of specific T (Th1, Th2, Th17) cells and immune pathways (IL-17, Toll-like receptor, TNF and JAK-STAT) as well as the cell differentiation pathway of osteoclast to the active RA pathogen–immune–inflammation responses. Intestinal microbiome dysbiosis might be the proximate underlying causes of RA onset and aggravation.

The intestinal epithelial cells cover the intestinal lumen and separate the gut microbiota from the immune system. The onset of RA may involve the interference between the internal immune system and external environmental influences on the intestinal microbiome. It is well known that IgA is the most abundant secretory Ig isotype in the gut and can be produced in both T-cell-dependent and -independent manners [22]. IgA shapes and maintains the intestinal microbial community and has a fundamental role in mucosal defence by coating and entrapping microorganisms and immobilizing the microbiome [22]. Interestingly, as a putative mechanism, one cause of RA onset manifested especially at gut mucosal surfaces via the key pathway of intestinal immune network for IgA production, resulting in differentiation of

autoreactive Th cells and other pathogenic pathways. Commensals with high IgA coating were found to be specifically enriched in patients with IBD, colonization of which was capable of inducing Th17 mucosal immunity [23]. Periodontitis is more prevalent in RA and is associated with high levels of ACPA [24]. It was reported that germ-free mice are protected against experimental arthritis, further indicating the imbalance of microbiome in the gut as the culprit responsible for RA [23]. While the precise mechanisms have not been fully understood, it is likely that the commensal microbiome may induce production of pro-inflammatory cytokines, and facilitate Th17 differentiation and the subsequent immune signalling pathways, such as the TNF signalling pathway, TCR pathway and Toll-like receptor pathway [25]. Several of the down-regulated DEmiRs (hsa-miR-1237-3p [26, 27], -125a-3p [28] and -197-3p [29, 30]) identified in this study are important regulators of the key genes (CD28, CD86 and HLA-DMB) in the pathway of intestinal immune network for IgA production. Other than the pathway of Toll-like receptor [25, 31], TNF [32], JAK-STAT [33] and osteoclast differentiation [34] are also involved in the onset and assistance of RA. At least three DEmiRs (hsa-miR-125a-3p, -197-3p and -1237-3p) are key regulators of these genes. Target genes of hsa-miR-575, -191-3p, -1237-3p, -6865-3p, -6886-3p and -4436b-5p were identified by HITS-CLIP. Hsa-miR-575 could be highly related to cell pathologic behaviours and may promote RA-FLS tumour-like behaviours regulated by LncRNA Gastric adenocarcinoma predictive long intergenic noncoding RNA (GAPLINC) [35]. miR-191-3p facilitates tetraploid growth by enhancing mitogenic signalling pathways [36].

Therefore, the function of the downregulated DEmiRs mainly involves the immunity mechanism of the onset of RA and aggravation of the disease. It implicates the intestinal dysbiosis–infection–inflammation–immune network as a key pathogen of the active RA.

The characterization of the up-regulated DEmiRs and miR interactome

In the serum of NARAPs, three DEmiRs (hsa-miR-187-5p, -4532, -4516) had higher levels than in the HCs. There have been no previous reports about the expression of hsa-miR-187-5p, -4532 and miR-4516 in serum or tissues in RA patients so far. Virus infection is one of the major environmental risk factors for RA development. Several mechanisms have been associated with the induction of autoimmunity by EBV. EBVs infect B cells possibly result in proliferation, enhanced antibody production and formation of immune complexes [31]. HBV infection was found to probably be involved in RA via p53 signalling pathway regulated by CDK6, TP53 and some miRs including miR-187-5p, -4516 and -6886-3p (evidence of CLIP) [37, 38], while RA patients with Toxoplasmosis infection were regulated miR-4516 and its target gene, Mitogen-activated protein kinase 13 and STAT3 with evidence of luciferase reporter assay [39]. Some other miRs, such as miR-155, have been reported to have a higher expression level in RA and may modulate the downstream signals involved in tissue damage or even have a protective function by downregulating the expression of certain MMPs, thus alleviating tissue damage [40]. Therefore, the function of the upregulated DEmiRs may be involved mainly in comorbidities and complications (e.g. EBV susceptibility, tumour susceptibility) of RA [40–43].

Limitations

Although three miRs (has-miR-125a [44], -197 [29] and -575 [35]) previously reported to play diverse biologic roles in RA were also identified as DEmiRs in the NARAPs, several other miRs previously associated with RA, such as miR-101-3p, miR-365, miR-144-5p, etc. [14], were not detected in the present study. This may be due not only to the differences in the patients included in these studies in terms of race, gender, drug treatment history, complications with other diseases, etc., but also to the relatively modest sample size of both NARAPs and HCs in the current study. Future studies are needed to verify the results of intestinal immunity and microbiome in larger populations of both HCs and RA patients. Further in-depth analysis of the cell specificity in the miR interactomes, especially the subtypes of immune cells such as lymphocytes, may uncover specific functions and mechanisms of the pathogen–inflammation–immune network in the context of the initial environments.

Summary

This study identified several serum DEmiRs as potential biomarkers for RA. Our predictive bioinformatics analysis suggested that downregulated miRs may play a fundamental role in intestinal microbiome and human innate and adaptive immunity, while up-regulated miRs may influence infection and autoimmunity associated with comorbidities and complications. These findings not only represent an initial step towards characterizing the diverse landscape of miR–target interactions across normal and diseased human with RA, but also offer clues that may facilitate the translation of genetic studies of complex RA, particularly, the RA-specific miR interactome, into novel or refined pathogenic diagnosis and therapeutics of active RA patients.

Acknowledgements

D.G. and D.D.D. conceived and designed the experiments. D.G., Y.L., Xi Chen, X.Y. and F.M. carried out most of the experiments. Xu Chen performed the data acquisition and analysis. D.G., D.N., J.L., Z.J. and D.N. contributed reagents/materials tools. D.G. and D.D.D. wrote and finalized the manuscript.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

References

1 McInnes IB, Schett G. Pathogenetic insights from the treatment of rheumatoid arthritis. Lancet 2017;389: 2328–37.

2 Aletaha D, Smolen JS. Diagnosis and management of rheumatoid arthritis: a review. JAMA 2018;320:1360–72.

3 Aletaha D, Maa JF, Chen S *et al.* Effect of disease duration and prior disease-modifying antirheumatic drug use on treatment outcomes in patients with rheumatoid arthritis. Ann Rheum Dis 2019;78:1609–15.

4 Firestein GS. Pathogenesis of rheumatoid arthritis: the intersection of genetics and epigenetics. Trans Am Clin Climatol Assoc 2018;129:171–82.

5 McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365:2205–19.

6 Chung IM, Ketharnathan S, Thiruvengadam M, Rajakumar G. Rheumatoid arthritis: the stride from research to clinical practice. Int J Mol Sci 2016;17:900.

7 Nemtsova MV, Zaletaev DV, Bure IV *et al.* Epigenetic changes in the pathogenesis of rheumatoid arthritis. Front Genet 2019;10:570.

8 Paredes S, Taverner D, Ferré R *et al.* Micro-RNA differential expression shared between rheumatoid arthritis and acute myocardial infarction: an exploratory study. Clin Exp Rheumatol 2019;37:886–7.

9 Andonian BJ, Chou CH, Ilkayeva OR *et al.* Plasma MicroRNAs in established rheumatoid arthritis relate to adiposity and altered plasma and skeletal muscle cytokine and metabolic profiles. Front Immunol 2019;10: 1475.

10 Wittmann J, Jäck HM. microRNAs in rheumatoid arthritis: midget RNAs with a giant impact. Ann Rheum Dis 2011;70(Suppl 1):i92–6.

11 Stanczyk J, Pedrioli DM, Brentano F *et al.* Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. Arthritis Rheum 2008;58:1001–9.

12 Filková M, Aradi B, Senolt L *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.

13 Nakamachi Y, Kawano S, Takenokuchi M *et al.* MicroRNA-124a is a key regulator of proliferation and monocyte chemoattractant protein 1 secretion in fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum 2009;60:1294–304.

14 Moran-Moguel MC, Petarra-Del RS, Mayorquin-Galvan EE, Zavala-Cerna MG. Rheumatoid arthritis and miRNAs: a critical review through a functional view. J Immunol Res 2018;2018:1–16.

15 Chou CH, Shrestha S, Yang CD *et al.* miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. Nucleic Acids Res 2018; 46:D296–302.

16 Ule J, Hwang H-W, Darnell RB. The future of Cross-Linking and Immunoprecipitation (CLIP). Cold Spring Harb Perspect Biol 2018;10:a032243.

17 Helwak A, Tollervey D. Identification of miRNA-target RNA interactions using CLASH. Methods Mol Biol 2016; 1358:229–51.

18 Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.

19 Bader GD, Hogue CW. An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinformatics 2003;4:2.

20 Reimand J, Isserlin R, Voisin V *et al.* Pathway enrichment analysis and visualization of omics data using g:Profiler, GSEA, Cytoscape and EnrichmentMap. Nat Protoc 2019;14:482–517.

21 Hsu S-D, Huang H-Y, Chou C-H *et al.* Integrated analyses to reconstruct microRNA-mediated regulatory networks in mouse liver using high-throughput profiling. BMC Genomics 2015;16:S12.

22 Li B, Selmi C, Tang R, Gershwin ME, Ma X. The microbiome and autoimmunity: a paradigm from the gut-liver axis. Cell Mol Immunol 2018;15:595–609.

23 Palm NW, de Zoete MR, Cullen TW *et al.* Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. Cell 2014;158:1000–10.

24 Terao C, Asai K, Hashimoto M *et al.* Significant association of periodontal disease with anti-citrullinated peptide antibody in a Japanese healthy population-The Nagahama study. J Autoimmun 2015;59:85–90.

25 Sujitha S, Rasool M. MicroRNAs and bioactive compounds on TLR/MAPK signaling in rheumatoid arthritis. Clin Chim Acta 2017;473:106–15.

26 Garcia-Lacarte M, Martinez JA, Zulet MA, Milagro FI. Implication of miR-612 and miR-1976 in the regulation of TP53 and CD40 and their relationship in the response to specific weight-loss diets. PLoS One 2018;13: e0201217.

27 Zheng ZF, Su HF, Zou Y, Peng Z, Wu SX. Expression profiles of microRNAs in radioresistant esophageal cell line. Zhonghua Yi Xue Za Zhi 2011;91:639–42.

28 Fujii S, Miura Y, Fujishiro A *et al.* Graft-versus-host disease amelioration by human bone marrow mesenchymal stromal/stem cell-derived extracellular vesicles is associated with peripheral preservation of naive T cell populations. Stem Cells 2018;36:434–45.

29 Cuppen BVJ, Rossato M, Fritsch-Stork RDE *et al.*; all SRU investigators. Can baseline serum microRNAs predict response to TNF-alpha inhibitors in rheumatoid arthritis. Arthritis Res Ther 2016;18:189.

30 Lerman G, Sharon M, Leibowitz-Amit R, Sidi Y, Avni D. The crosstalk between IL-22 signaling and miR-197 in human keratinocytes. PLoS One 2014;9:e107467.

31 Sternbæk L, Draborg AH, Østerlund MT *et al.* Increased antibody levels to stage-specific Epstein-Barr virus antigens in systemic autoimmune diseases reveal a common pathology. Scand J Clin Lab Invest 2019;79:7–16.

32 Cuppen BV, Jacobs JW, Ter BEJ *et al.* Necessity of TNF-alpha inhibitor discontinuation in rheumatoid arthritis is predicted by smoking and number of previously used biological DMARDs. Clin Exp Rheumatol 2017;35:221–8.

33 Malemud CJ. The role of the JAK/STAT signal pathway in rheumatoid arthritis. Ther Adv Musculoskelet Dis 2018;10:117–27.

34 Harshan S, Dey P, Ragunathan S. Effects of rheumatoid arthritis associated transcriptional changes on osteoclast differentiation network in the synovium. PeerJ 2018;6:e5743.

35 Mo BY, Guo XH, Yang MR. Long non-coding RNA GAPLINC promotes tumor-like biologic behaviors of fibroblast-like synoviocytes as MicroRNA sponging in rheumatoid arthritis patients. Front Immunol 2018;9:702.

36 Vittoria MA, Shenk EM, O'Rourke KP *et al.* A genome-wide microRNA screen identifies regulators of tetraploid cell proliferation. Mol Biol Cell 2018;29:1682–92.

37 Hou X, Liang Y, Chen J *et al.* Expression profiling of cellular microRNA in asymptomatic HBsAg carriers and chronic hepatitis B patients. Biomed Res Int 2017;2017:1–17.

38 Li H, Wang Z, Jiang M *et al.* The oncoprotein HBXIP promotes human breast cancer growth through down-regulating p53 via miR-18b/MDM2 and pAKT/MDM2 pathways. Acta Pharmacol Sin 2018;39:1787–96.

39 Chowdhari S, Saini N. hsa-miR-4516 mediated downregulation of STAT3/CDK6/UBE2N plays a role in PUVA induced apoptosis in keratinocytes. J Cell Physiol 2014;229:1630–8.

40 Long L, Yu P, Liu Y *et al.* Upregulated microRNA-155 expression in peripheral blood mononuclear cells and fibroblast-like synoviocytes in rheumatoid arthritis. Clin Dev Immunol 2013;2013:1–10.

41 Wells PM, Williams FMK, Matey-Hernandez ML, Menni C, Steves CJ. RA and the microbiome: do host genetic factors provide the link. J Autoimmun 2019;99:104–15.

42 Wu X, He B, Liu J *et al.* Molecular insight into gut microbiota and rheumatoid arthritis. Int J Mol Sci 2016;17:431.

43 Wu YH, Liu W, Xue B *et al.* Upregulated expression of microRNA-16 correlates with Th17/Treg cell imbalance in patients with rheumatoid arthritis. DNA Cell Biol 2016;35:853–60.

44 Churov AV, Oleinik EK, Knip M. MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. Autoimmun Rev 2015;14:1029–37.