Original Article
Characterization of long non-coding RNA-mediated competing endogenous RNA network to reveal potential long non-coding RNA biomarkers in rheumatoid arthritis patients under anti-TNF treatment

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Abstract: Objective: We designed to identify long non-coding RNA (lncRNA) signatures that predicted the therapy outcomes in rheumatoid arthritis (RA) via characterizing lncRNA-mediated competing endogenous RNA network (LMCN). Methods: Hypergeometric test was used to detect the competing lncRNA-mRNA interactions, following by co-expression analysis for the competing lncRNA-mRNA interactions relying on pearson correlation coefficient (PCC). The PCC absolute value of one interaction was defined as the weight value of one edge. Using the weight value threshold of 0.6, we established a highly competitive LMCN. To analyze the network organization, we conducted degree distribution of LMCN. Moreover, to predict the function of significant lncRNAs, we implemented the pathway analysis for its mRNA neighbors in the LMCN. Finally, we employed Biclique algorithm to extract competing modules from the LMCN. Results: Relying on a weight value > 0.6, a high-competing LMCN was constructed, which covered 50 lncRNAs, 824 mRNAs, and 926 competing endogenous (ceRNA) interactions. Based on degrees > 40, a total of 7 hub genes were identified, including SNHG12, C1RL-AS1, TTTY15, MALAT1, TAPT1-AS1, LINC00476, and LINC00649. Remarkably, a hub lncRNA LINC00476 was involved in the signaling-associated pathways (phosphatidylinositol signaling system, Ras signaling pathway, Rap1 signaling pathway, and TNF signaling pathway). Overall, we extracted 1 synergistic, competitive module containing 30 nodes. Significantly, one lncRNA LINC00476 in the LMCN was also the hub within the module. Conclusion: Our exploratory study indicates that the obtained lncRNA signatures provide novel information to better understand the mechanisms of action of anti-TNF treatment in RA patients.

Keywords: Rheumatoid arthritis, long non-coding RNA, lncRNA-mediated ceRNA network, module

Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune inflammatory disorder, which is characterized by synovitis proliferation, erosion of the marginal bone, inflammatory joint fluid, leucocyte extravasation, and systemic inflammatory manifestations. Worriedly, RA is highly associated with complications, disability, and society and economic cost [1, 2]. Anti-tumor necrosis factor (anti-TNF) agents are the most effective therapeutic schemes in RA, which serve to neutralize the signaling of pro-inflammatory cytokine [3, 4]. However, about 30% of RA patients treated by anti-TNF agents fail to obtain clinical improvement, exposing them to unnecessary side effects when their disease worsens [5, 6]. The risk for adverse effects and high costs in the non-response to anti-TNF patients have driven the seek for predictive signatures which are able to predict therapy outcome.

Though more and more efforts have been done to extract biomarkers (for example, genes coding TNF, TNF receptors, and Fc receptor IIIA) to predict response to anti-TNF in RA [7-9], predictive value has not been observed among independent cohorts. Fortunately, in recent years, many researchers have emphasized the importance of non-coding RNAs (ncRNAs) in several

biological processes [10]. Among the ncRNAs, the long ncRNAs (lncRNAs) is one of the largest gene families in the human genome [11]. Though the specific roles of lncRNAs are largely unknown, several studies have documented their key functions in many biological processes including post-transcriptional regulation and genomic imprinting [12, 13]. Of note, many studies have demonstrated that lncRNAs are involved in the occurrence and progression of RA. Two lncRNAs, FAM66C and Hotair, have been identified to regulate the immune functions in RA [14, 15]. In addition, Messemaker et al. [16] have demonstrated that a novel lncRNA C5T influences the transcript level of gene *C5* which is shown to be associated with RA pathogenesis. Although the molecular roles of many lncRNAs related to RA are yet unravelled, in-depth functional and mechanistic characterization of lncRNAs which predict the response to anti-TNF therapies in RA appears to be an emerging area and a promising opportunity. Thus, uncovering the lncRNA signatures and their functions in RA patients treated by anti-TNF agents is challenging.

It is noteworthy that characterization of lncRNA functions are typically implemented relying on a 'guilt by association' strategy [17, 18]. In recent years, several studies have implicated lncRNAs function as competing endogenous RNAs (ceRNAs) via harboring miRNA response elements to regulate expression level of mRNAs [19, 20]. Systematic analyses of lncRNA-associated ceRNA network have been conducted in cancer [21, 22], and the results have revealed the functional significance of lncRNA-associated ceRNA network in diseases. Nevertheless, no studies have been reported the complexity and behavior of lncRNA-associated ceRNA network in RA patients treated with anti-TNF agents.

In the current analysis, in order to better predict the clinical response to anti-TNF therapy in RA, we designed to identify the candidate functional lncRNA biomarkers on the basis of establishing a lncRNA-mediated ceRNA network (LMCN). The constructed LMCN offers important clues for understanding the key roles of ceRNA mediated gene regulatory network and lncRNA signatures in predicting the response to anti-TNF in RA.

Materials and methods

Collecting miRNA-target interactions

StarBase v2.0 (http://starbase.sysu.edu.cn/) [23] offers high-quality miRNA-target interactions which are experimentally confirmed and are deposited in published studies. Herein, in our work, we derived the experimentally validated miRNA-mRNA interactions and lncRNA-miRNA interactions from StarBase 2.0 [23].

Data collection

The mRNA and lncRNA expression data of RA treated with anti-TNF were derived from the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/), which was accessible through E-GEOD-33377 [24]. The data were curated from the platform of A-AFFY-143-Affymetrix GeneChip Human Exon 1.0 ST Array version 1, [HuEx-1_0-st-v1]. Microarray data of E-GEOD-33377 included 42 RA patients, including 24 patients showing no response to the anti-TNF treatment, and 18 patients responding well to anti-TNF treatment.

Prior to analysis, we pre-processed the raw data. Specifically, the probe sets were aligned to the gene symbols, and a total of 14,451 genes were extracted. After that, these 14,451 genes were mapped to the lncRNA-miRNA interactions and miRNA-mRNA interactions. Eventually, expression profiles of 51 lncRNAs and 8471 mRNAs were selected. Next, the interactions including any genes of 51 lncRNAs and 8471 mRNAs were respectively extracted from the lncRNA-miRNA interactions and miRNA-mRNA interactions. Overall, 598 lncRNA-miRNA interactions and 265,782 miRNA-mRNA interactions were identified.

Identifying potential ceRNA interactions

In our study, the hypergeometric test was used to select competing lncRNA-mRNA interactions, which can measure the significance of the common miRNAs between each mRNA and lncRNA. Assuming that the genome owned a number of H miRNAs, out of which, E and F were the numbers of miRNAs connected with the mRNA and lncRNA, and G was common miRNA number shared by lncRNA and mRNA. To assess the enrichment significance of the common miRNAs, we calculated the P values:
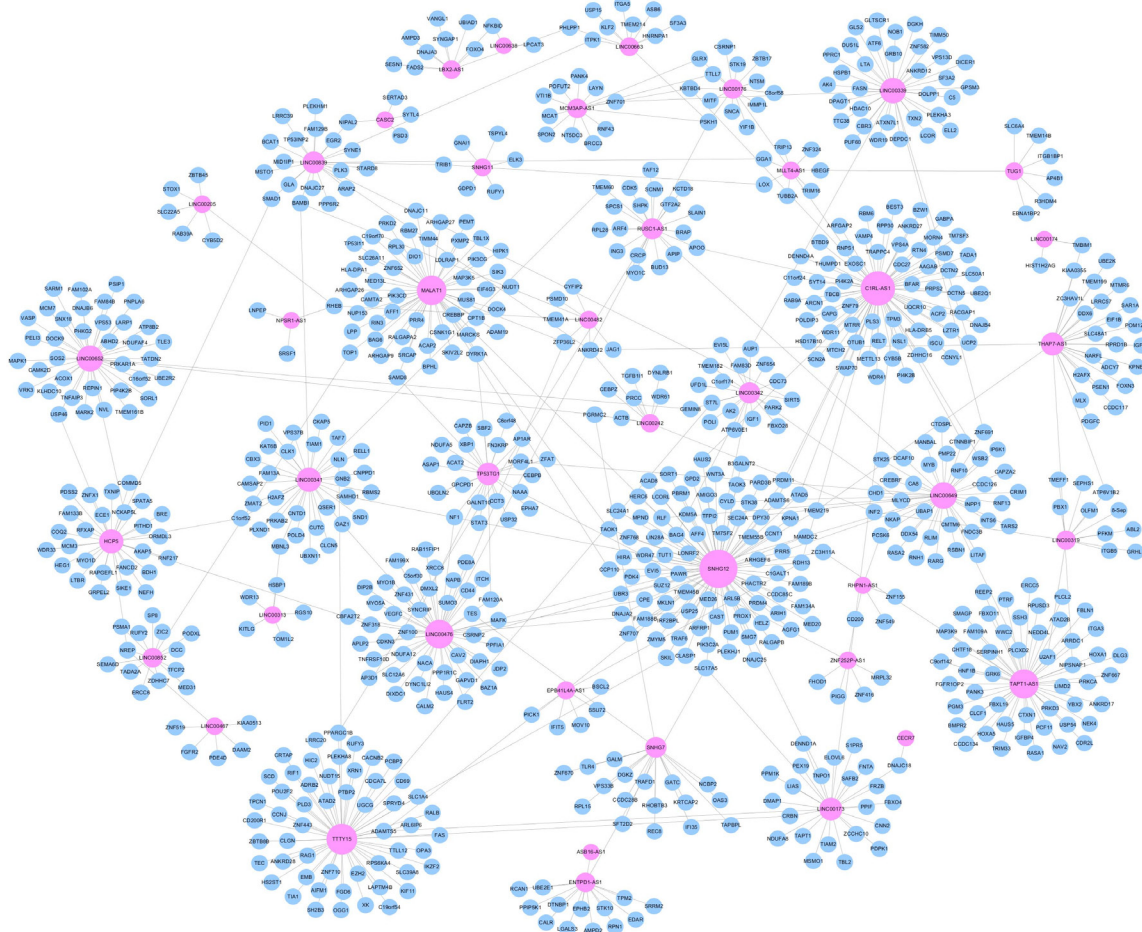
Figure 1. Construction of the highly competitive long non-coding RNAmediated competitive endogenous RNA network (LMCN). The LMCN covered 50 lncRNAs, 824 mRNAs, and 926 ceRNA interactions. Blue nodes were mRNAs, and pink nodes denoted lncRNAs.

$$P = 1 - \sum_{t=0}^{G} \frac{\binom{E}{t}\binom{H-E}{F-t}}{\binom{H}{F}}$$

Then, false discovery rate (FDR) was applied to adjust the original *P* values relying on Benjamini & Hochberg method [25]. A FDR < 0.01 was acted as the cut-off threshold.

Construction of a highly competitive LMCN

To evaluate the co-expression probability of lncRNA-mRNA pairs, we calculated the PCC on the basis of the expression of the competing lncRNA-mRNAs pairs according to the equation described below:

$$P_{m,n} = cov\ (m, n)/\sigma_m\sigma_n$$

Where cov (m, n) was the covariance of variables m and n, while σ_m and σ_n respectively

stood for the standard deviations (SD) for m and n. The weight values in our work were determined as the PCC absolute value of one interaction and only edges having correlations higher than 0.6 were kept to establish the LMCN.

Network illustration and topological analysis for LMCN

In order to visualize the LMCN, we utilized Cytoscape tool (http://cytoscape.org/) [26] to illustrate the highly competitive LMCN. The topological centrality indexes (including degree, closeness and betweenness) are broadly employed to analyze the properties of network [27]. Of which, degree is the simplest index. As documented, degree is the number of links that one node connects with other nodes [28]. In the current work, the degree distribution of all
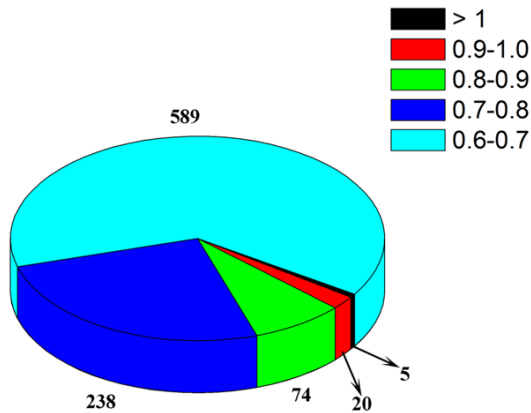
Figure 2. Pie chart showing the weight distribution of interactions. The weight values were classified into the following groups: > 1, 0.9~1.0, 0.8~0.9, 0.7~0.8, and 0.6~0.7.

nodes in LMCN was analyzed and the nodes having degrees higher than 40 were regarded as hubs.

Extracting synergistic, competing lncRNA modules

Because LMCN can supply an overall view of all competing lncRNA-mRNA interactions which enable illumination of the regulatory features of the lncRNAs, the sub-networks demonstrate a more detailed picture of how lncRNAs synergized with the competing mRNAs. Biclique approach was derived from the website of the Computational Biology Laboratory in the Department of Computer Science, Iowa State University (http://genome.cs.iastate.edu/supertree/download/biclique/). Significantly, Biclique method was employed in a former study of ceRNA networks [19] to detect synergistic, competing modules in the LMCN. Thus, herein, we also used the Biclique algorithm to identify the synergistic competing modules from the LMCN.

Analysis of functional annotation

As reported, Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway) is a database used to systematically analyze gene functions [29]. In our study, with the goal of revealing the underlying biological process of lncRNAs, we used DAVID tool to implement pathway analyses for genes in LMCN according to the 'guilt by association' strategy. Fisher's test was utilized to detect the

significant pathways which were selected using the criteria of FDR < 0.05.

Results

Establishment of a highly competitive LMCN

In order to construct the highly competitive LMCN, we firstly used the hypergeometric test to identify candidate lncRNA-mRNA competing pairs through evaluating the significance of the common miRNAs between each lncRNA-mRNA pair. Consequently, based on FDR < 0.01, 51 lncRNAs, 8125 mRNAs, and 34,586 ceRNA interactions were extracted. Then, we applied PCC to measure the co-expression probability of lncRNA-mRNA pairs. Relying on a weight value higher than 0.6, a high-competing LMCN was constructed, which covered 50 lncRNAs, 824 mRNAs, and 926 ceRNA interactions. The LMCN was displayed in **Figure 1.** From this figure, we found that the lncRNAs were in the central area of the network, but the mRNAs were typically in the outside layer (**Figure 1**). we also found that nodes interacted with each other, but the weight scores were different. The interactions with higher weight values might be more important than the others for RA treated by anti-TNF. The majority of interactions were distributed in the weight range of 0.6~0.7 (589), followed by the range between 0.7~0.8 (238), 0.8~0.9 (74), 0.9~1.0 (20), and > 1 (5). The weight distribution of the interactions in LMCN was shown in **Figure 2**.
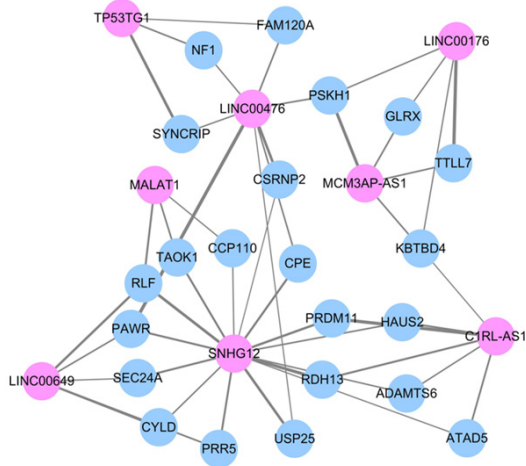
Degree properties of the LMCN

To explore the organization of the LMCN in RA patients treated with anti-TNF agents, we investigated the degree distribution of LMCN. The results of the degree distribution of the entire network (R^2 = 0.9996) demonstrated power law distributions (**Figure 3A**), which demonstrated that the LMCN was a scale-free network. The investigation implicated that the LMCN was parallel to biological networks and was well organized by a core set of lncRNA-mRNA competing principles into structured instead of random networks. Commonly, a higher degree suggested that the node was a hub which participated in more ceRNA interactions. In **Figure 3B**, we found that the degree distribution of the lncRNAs was greater than that of the mRNAs, which further demonstrated that although lncRNAs did not code for pro-
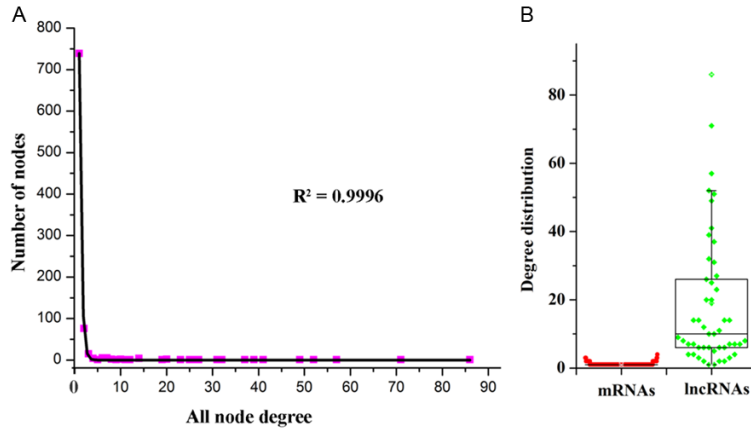
Figure 3. Degree analysis of the LMCN. A: The degree distributions of the entire network reveal a power-law distribution. B: Box-type scatterplot suggested that the node degree distribution of the lncRNAs was higher than that of the mRNAs.

Figure 4. A synergistic, competing module identified with Biclique algorithm.

teins, they displayed more specific degree property than mRNAs in the LMCN. According to the features of the lncRNAs within the LMCN, we supposed that some hub nodes were risk lncRNAs for RA. This characteristic was employed to extract modules in the network and deduce which nodes were prognostic factors. To identify the hub nodes in the LMCN, the degrees of all nodes within the LMCN were ranked in a descending order on the basis of their degree distribution. Relying on degrees more than 40, a total of 7 hub genes were identified, including SNHG12 (degree = 86), C1RL-AS1 (degree = 71), TTTY15 (degree = 57), MALAT1 (degree = 52), TAPT1-AS1 (degree =

51), LINC00476 (degree = 49), and LINC00649 (degree = 41).

Extracting synergistic, competing lncRNA modules

In order to investigate the modularity characteristics of the LMCN, the Biclique method was employed in the present work. Overall, we extracted 1 synergistic, competitive module containing 30 nodes, as exhibited in **Figure 4**. We observed that lncRNA LINC-00476 competed with 6 mRNAs (NF1, FAM120A, SY-NCRIP, CSRNP2, PSKH1, and PAWR) and 5 lncRNAs (LINC-00176, MCM3AP, TP53TG1, SNHG12 and LINC00649) in a 30-ceRNA module (**Figure 4**), demonstrating its significant functions in RA treated with anti-TNF agents. These results also suggested that there were synergistic regulatory effects among the 6 lncRNAs. Moreover, lncRNA SNHG12 competed with 12 mRNAs and 4 lncRNAs (LINC00476, MALAT1, C1RL-AS1, and LINC00649) in this module (**Figure 4**). Significantly, two lncRNAs SNHG12 and LINC00476 in the LMCN were also the hubs within the synergistic, competing lncRNA module.

Analysis of functional annotation

To further reveal the potential biological functions of lncRNAs in the response to anti-TNF agents in RA, KEGG pathway enrichment analysis of mRNAs in the LMCN was conducted. Based on FDR < 0.05, we found that the mRNAs were remarkably enriched in 9 pathways which were mainly involved in 4 functional clusters including neuroactive ligand-receptor interaction, signaling-related pathways (phosphatidylinositol signaling system, Ras signaling pathway, Rap1 signaling pathway, and TNF signaling pathway), metabolic process associated with fatty acid and choline, and proteoglycans in cancer. Remarkably, a hub lncRNA LINC00476 was involved in the signaling-associated pathways (phosphatidylinositol signaling system, Ras signaling pathway, Rap1 signaling pathway, and TNF signaling pathway). Moreover, another

Table 1. Significant pathways based on false discovery rate (FDR) < 0.05

Pathway IDs	Pathway terms	FDR values
hsa04080	Neuroactive ligand-receptor interaction	0.006506
hsa04070	Phosphatidylinositol signaling system	0.024879
hsa04014	Ras signaling pathway	0.049537
hsa04015	Rap1 signaling pathway	0.049537
hsa04668	TNF signaling pathway	0.049537
hsa01212	Fatty acid metabolism	0.049537
hsa04960	Aldosterone-regulated sodium reabsorption	0.049537
hsa05231	Choline metabolism in cancer	0.049537
hsa05205	Proteoglycans in cancer	0.049537

hub lncRNA SNHG12 was significant related to the pathway of posphatidylinositol signaling system. Specific information was shown in **Table 1**.

Discussion

The development of TNF blocking agents has resulted in major advances in the management of RA. Effectiveness of anti-TNF treatment in RA has recently modified the therapeutic approach of this disorder. Nevertheless, despite extensive investigation, the molecular mechanisms of anti-TNF action remain poorly understood [30]. Previously, significant efforts have been made to extract gene markers to predict the response to anti-TNF therapy in PA [7-9], but the biomarkers often had little overlap. With the aim of shedding light of the molecular processes participating in the beneficial effects of anti-TNF treatment in good responder RA patients, we constructed lncRNA-associated ceRNA network based on the paired miRNA, mRNA, lncRNA expression data. To our knowledge, this is the first study exhibiting the functional significance of reported lncRNA-associated ceRNA network and identified the lncRNA signatures for predicting anti-TNF response in RA.

In our study, two hub lncRNAs, SNHG12 and LINC00476 in the LMCN were also the hubs within the synergistic, competing lncRNA module. SNHG12 has been reported to be involved in cell proliferation, cell migration, angiogenesis and carcinogenesis [31]. The LINC00476 has been predicted to be in pathways connected with cartilage and bone development [32]. Through the literature review, the functions and roles of SNHG12 and LINC00476 in RA have not been yet reported.

In order to predict the function of significant lncRNAs, we implemented the KEGG pathway analysis for its mRNA neighbors in the LMCN. We observed that inferred functions of these lncRNAs were involved in several signaling-related pathways (phosphatidylinositol signaling system, Ras signaling pathway, Rap1 signaling pathway, and TNF signaling pathway). More significantly, in our study, a hub lncRNA LINC00476 was involved in these signaling pathways. Based on the published studies, these signaling pathways are associated with immune response. Specially, phosphatidylinositol signaling is a key controller of TH17 cell differentiation, and these TH17 cells can produce IL17 which is a main driver of inflammation and autoimmunity [33, 34]. The phosphatidylinositol signaling system pathway is a component of the adaptive immune response and is essential for the maintenance of self-tolerance [35]. RAP proteins are small GTPases of the RAS family, which are expressed in many cell types. Significantly, Ras activation is required for p38 and ERK activation and MSC osteogenesis [36]. The activated ERK pathway has been reported to regulate the inflammatory response [37]. TNF-alpha is the prime inflammatory mediator, and the inflammatory factors have been demonstrated to be closely associated with the RA pathogenesis [38, 39].

Significantly, Thomson et al. [40] have demonstrated that the blood of anti-TNF non-responders may generally be associated with cytokine signaling involved in inflammation, and also might participate the specific metabolic activities related lipolysis and fatty acid oxidation. A former study has suggested that responders to anti-TNF treatment are characterized by the abnormal expression of inflammatory genes in synovial tissue [41]. Moreover, the aberrant expression of inflammatory genes in responders faster than in non-responders [42]. Baarsen et al. [43] have shown that TNF treatment causes dys-expression of genes in diverse immune related pathways including inflammation. Demonstrated herein, we infer that a hub lncRAN LINCA00476 might play impor-

tant roles in predicting the anti-TNF treatment outcome of RA patients, partially though regulating the inflammatory related signaling pathways.

However, we must take into consideration of several limitations in our study. Firstly, our study involved in the relatively small samples. Secondly, the dataset used in our analysis were downloaded from the public database, not generated by us. Moreover, there was no other independent data to verify our results because of the limitation of available lncRNA expression. Additionally, the results were obtained according to bioinformatics-based-scanning, yet were not verified using biological experiments. Thus, it is urgent to do experimental studies to validate our obtained findings.

Taken together, our study successfully extracted several lncRNA biomarkers (especially LINC00476) predictive of anti-TNF treatment outcome in RA patients. These lncRNA signatures, if verified prospectively, may have important implications for the identification of clinical outcomes of TNF blocking treatment in RA and can be viewed as a start to establish a prediction model for anti-TNF treatment outcome.

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Our study were not involved in human participants or animals.

Disclosure of conflict of interest

None.

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