Expression signature of inflammation-associated long non-coding RNAs in adult-onset Still's disease

C.-A. Yang[1,2], P.-K. Chen[2,3,4], J.-L. Lan[2,4,5], C.-K. Chang[2,3,4], J.-G. Chang[2,6], S.-H. Chang[2,4], C.-C. Lin[2,7], D.-Y. Chen[2,3,4,7]

[1]Division of Laboratory Medicine, China Medical University Hsinchu Hospital, Zhubei City; [2]College of Medicine, China Medical University, Taichung; [3]Translational Medicine Laboratory, Rheumatology and Immunology Centre, China Medical University Hospital, Taichung; [4]Rheumatology and Immunology Centre, China Medical University Hospital, Taichung; [5]Rheumatic Diseases Research Centre, China Medical University Hospital, Taichung; [6]Epigenome Research Centre, China Medical University Hospital, Taichung; [7]Ph.D. Program in Translational Medicine and Rong Hsing Research Centre for Translational Medicine, National Chung Hsing University, Taichung, Taiwan.

Chin-An Yang, MD, PhD
Po-Ku Chen, PhD
Joung-Liang Lan, MD
Ching-Kun Chang, PhD
Jan-Gowth Chang, MD
Shih-Hsin Chang, MD
Chi-Chien Lin, PhD
Der-Yuan Chen, MD, PhD

Please address correspondence to:
Der-Yuan Chen,
Rheumatology and Immunology Centre, China Medical University Hospital, No. 2 Yude Road, Taichung, 40447 Taiwan.
E-mail: dychen1957@gmail.com

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ABSTRACT

Objective. *Adult-onset Still's disease (AOSD) is a rare and complex inflammatory disease with unclear immunopathogenesis. This study aims to investigate the expression signature of inflammation-associated long non-coding RNAs (lncRNAs) in AOSD and to evaluate its utility for disease diagnosis and prognostication.*

Methods. *Expression levels of lncRNAs* MIAT, THRIL, NTT, RMRP, PACERR *and* NEAT1 *in peripheral blood mononuclear cells (PBMCs) from treatment-naïve AOSD patients and healthy donors were assessed by quantitative real-time PCR and logistic regression analysis.*

Results. *A diagnostic scoring algorithm was built based on the expression pattern of* MIAT, THRIL *and* RMRP, *which could differentiate AOSD from patients with rheumatoid arthritis, systemic lupus erythematosus, or sepsis. Our score could also predict the need of biologics in AOSD treatment. We further followed up ten AOSD patients and found that the expression of* NEAT1 *was positively correlated with the expression levels of* MIAT, THRIL *and* RMRP *after treatment. In poly(I:C)-stimulated THP-1 cell and primary monocytes,* MIAT *up-regulation coupled with* THRIL *down-regulation was similar to the expression pattern observed in AOSD.*

Conclusion. O*ur study provides an AOSD diagnostic scoring system based on the expression signature of* MIAT, THRIL *and* RMRP. *Further investigations are needed to uncover the mechanisms of lncRNA dysregulation in AOSD.*

Introduction

Adult-onset Still's disease (AOSD), an inflammatory disorder with complex aetiologies, is characterised by fever, rash, arthritis, multisystemic involvement, and elevated levels of acute phase reactants (1, 2). It has been reported that Th17-related cytokines and proinflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and IL-18 are involved in AOSD pathogenesis (3-6). Furthermore, virus infection (7), NLRP3-inflammasome activation (8), elevated amount of low-density granulocytes and neutrophil extracellular traps (NETs) (9), and increased circulating microRNA-134 were found to be associated with AOSD (10). Despite such evidence, diagnostic markers are still lacking for this complex and heterogeneous disease, and its diagnosis is usually made by clinical criteria (11).

Long non-coding RNAs (lncRNAs), the non-protein-coding transcripts greater than 200 nucleotides, have emerged as novel players in gene regulation (12, 13). LncRNAs are involved in transcription process and cellular response through RNA-DNA, RNA-RNA or RNA-protein interactions (12-15). LncRNAs play a critical role in innate and adaptive immunity (14-17). Furthermore, recent studies also revealed that lncRNAs can be induced in immune cells and act as the key regulators of inflammatory responses (16-18) and antiviral immunity (19-21). For example, *MIAT* (myocardial infarction associated transcript) is involved in the pathogenesis of various diseases, including myocardial infarction, microvascular dysfunction, and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (21-23). *THRIL* (TNF-α and hnRNPL-related immunoregulatory lincRNA) acts as a scaffold through interacting with heterogeneous nuclear ribonucleoprotein-L (hnRNPL), and this complex binds to TNF-α promoter to induce its transcription (24). *NTT* (non-coding transcript in T-cell), which is discovered in activated $CD4^+$T cells (25), has

been reported to regulate inflammation and contributes to the pathogenesis of rheumatoid arthritis (RA) (26). *RMRP* (RNA component of the mitochondrial RNA-processing endoribonuclease) promotes RORγtDDX5 assembly and is recruited to RORγt-occupied loci of critical genes implicated in the Th17 effector program (27). *PACERR* (p50-associated COX-2 extragenic RNA), is a positive regulator of COX-2 expression in macrophage-like cells after TLR4 activation (28). *NEAT1* (nuclear enriched abundant transcript-1) is essential for the formation of nuclear body paraspeckles, which facilitate IL-8 transcriptional activation (29).

We therefore had particular interests in the aforementioned six lncRNAs *MIAT*, *THRIL*, *NTT*, *RMRP*, *PACERR*, and *NEAT1*, which are related to immune regulation or inflammatory response that might be involved in pathogenesis of AOSD (3-8, 10). In this study, we aimed to investigate the lncRNA expression signature in AOSD patients and its potential value in disease diagnosis and prognostication.

Materials and methods

Study subjects

Fifty-six consecutive AOSD patients fulfilling the Yamaguchi criteria (11) were enrolled. Patients with infections, malignancies or other rheumatic diseases were excluded. The disease activity was assessed using a modified Pouchot score described by Rau *et al*. (30), with active AOSD defined as activity scores ≥4 (31). At the study entry, all patients were treated with nonsteroidal anti-inflammatory drugs with/without corticosteroids, but none received conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) or biologic therapy. Defined as in previous studies (31, 32), all the enrolled AOSD patients who were followed for at least one year were classified into two subtypes of disease course: a systemic subtype that includes monocyclic and polycyclic form, and the other chronic articular subtype. Twenty patients fulfilling the 2010 classification criteria for RA (33) and 20 patients fulfilling the 1997 revised criteria for SLE (34) were included as disease controls. A sepsis transcriptome cohort E-GEOD-32707 was also downloaded from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) for the evaluation of lncRNA expressions. Thirty-two age-matched healthy subjects were used as healthy controls (HC). The Institutional Review Board approved this study (CMUH108-REC1-099), and each participant's written consent was obtained according to the Declaration of Helsinki.

RNA extraction and quantitative real-time PCR (qRT-PCR) for lncRNAs

Peripheral blood mononuclear cells (PBMCs) were immediately isolated from venous whole blood using the Ficoll-Paque™ PLUS (GE Healthcare Biosciences, Illinois, USA) density gradient centrifugation. Total RNAs from PBMCs were extracted by TRIzol® Reagent (Sigma-Aldrich, Missouri, USA) and purified using a RNeasy MinElute Cleanup kit (QIAGEN, Germany) according to the manufacturer's instructions. High-Capacity cDNA Reverse Transcriptase Kit (ThermoFisher Scientific-Invitrogen, Massachusetts, USA) was used to reverse-transcribe 2μg RNA into cDNA. Human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression was used as an endogenous control. All primers were designed and synthesised by Genomics BioSci & Tech, Taipei, Taiwan. The qRT-PCR reactions were performed on the Roche LightCycler Instrument 480 using IQ^2 TaqMan Probe qPCR system (Thermo Fisher Scientific, Massachusetts, USA). Real-time PCR using 100ng cDNA was performed with one cycle of preincubation at 95°C for 30sec, 50 cycles of amplification (95°C for 10 secs, 60°C for 30sec, 72°C for 10sec), and a final cooling at 40°C for 30sec. The difference of expression in the target gene relative to averaged internal control gene was calculated using the comparative threshold cycle (Ct) method.

Gene primer sequences:

MIAT forward 5'-CTGGAGAGGGAGGCATCTAA-3', reverse 5'-AACTCATCCCCACCCACAC-3'; *THRIL* forward 5'- AACAGGTGCACGTTTCAGG-3', reverse 5'- TACACATGATGGGACCCAAA-3'; *NTT* forward 5'- CTTGGCCTAAAAGGGGATG-3', reverse 5'- GCACCTTTGGTCTCCTTCAC-3'; *RMRP* forward 5'- AGAAGCGTATCCCGCTGAG-3', reverse 5'- GAGAATGAGCCCCGTGTG-3'; *PACERR* forward 5'- TCCACGGGTCACCAATATAAA-3', reverse 5'- CGTCCCTGCAAATTCTGG-3'; *NEAT1* forward 5'- CTCTGACCCGAAGGGTAGG-3', reverse 5'-CTGGCAGCTTTGCTCCTG-3'; *GAPDH* forward 5'-AGCCACATCGCTCAGACAC-3', reverse 5'- GCCCAATACGACCAAATCC-3'.

In vitro cell studies

The human monocytic cell lines, THP-1 cells (ATCC TIB-202; American Type Culture Collection, Rockville, Md.), were grown in lipopolysaccharide (LPS)-free RPMI medium (Gibco, ThermoFisher Scientific, USA) supplemented with 10% fetal bovine serum in an incubator containing 5% CO_2 at 37°C. One million cells cultured in LPS-free RPMI medium were treated with TLR3 ligand poly(I:C) (InvivoGen, California, USA) or TLR4 ligand LPS (Sigma-Aldrich, Merk, Darmstat, Germany) for 4 hours and 24 hours respectively. PBMCs derived from healthy controls were rested overnight in RPMI medium at 37°C incubator, and were separated into the adherent monocytes and the suspended lymphocytes. Poly(I:C) and LPS stimulation experiments were also performed on primary monocytes and lymphocytes. RNAs were then extracted from THP-1 cells, primary human monocytes and lymphocytes for further qRT-PCR analyses. The difference in expression of the target gene relative to averaged internal control gene was calculated using the comparative threshold cycle (Ct) method and evaluated by ΔCT ($CT^{lncRNA} - CT^{GAPDH}$). The fold of expression of lncRNAs was calculated by 2^-ΔΔCT [($CT^{lncRNA} - CT^{GAPDH}$) - median ΔCT of control replicates].

Statistical analysis

Multi-nominal regression analysis using the lncRNA expression levels (dCT) as variables for diagnosing AOSD was performed by using the generalised linear model (glm) function of R software v.

3.6.0 (R Foundation for Statistical Computing, Vienna, Austria) (36). The lncRNA expression signature of each sample from AOSD patients and HC were plotted on 3D-scatterplots by using R software to visualise key variables separating AOSD patients from controls. The AOSD cohorts were randomly divided into a test dataset (80% of the samples) and a validation dataset (20% of the samples), and a confusion matrix was built using the selected variables to summarise the performance of the classification algorithm (R software, glm function). AOSD prediction score was established by the combination of variables best differentiating AOSD and controls. The correlation coefficient was obtained through the nonparametric Spearman's rank correlation test. Receiver operating characteristic (ROC) curve analysis was performed to determine the area under ROC curve (AUC), sensitivity, specificity, and accuracy using MedCalc v. 14. Mann-Whitney U-test was used to evaluate the difference of scoring values between two groups via GraphPad Prism v. 8 (La Jolla, CA, USA).

Table I. Demographic data and clinical characteristics of patients with AOSD, RA, SLE, and healthy controls (HC)[#].

Characteristics	AOSD (n=56)	RA (n=20)	SLE (n=20)	HC (n=32)
Age at study entry, years	38.6 ± 11.9***	51.3 ± 11.2	39.6 ± 10.6**	38.3 ± 8.9
Female proportion, n (%)	45 (80.4%)	17 (85.0%)	18 (90.0%)	24 (75.0%)
Disease duration, years	1.9 ± 1.2***††	6.4 ± 3.2	4.8 ± 1.9*	NA
Disease activity scores	5.1 ± 1.2[a]	4.7 ± 1.2[b]	11.6 ± 3.3[c]	NA
ESR, mm/1st hour	45.2 ± 26.2†	36.0 ± 13.0	24.9 ± 7.1	NA
CRP, mg/dl	4.07 ± 4.28	2.13 ± 1.55	NA	NA
The used csDMARDs during follow-up period				
Prednisolone	50 (89.3%)	15 (75.0%)	17 (85.0%)	NA
Methotrexate	38 (67.9%)	17 (85.0%)	0 (0.00%)	NA
Hydroxychloroquine	31 (55.4%)	10 (50.0%)	19 (95.0%)	NA
Sulfasalazine	8 (14.3%)	13 (65.0%)	0 (0.00%)	NA
Azathioprine	2 (3.6%)	0 (0.00%)	6 (30.0%)	
Cyclosporine	10 (17.9%)	3 (15.0%)	2 (10.0%)	NA
The used biologics during follow-up period				
TNF-α inhibitors	0 (0.0%)	2 (10.0%)	0 (0.0%)	NA
IL-6R inhibitor	6 (10.7%)	1 (5.0%)	0 (0.0%)	NA

[#]Data presented as mean ± SD or number (percentage).
NA: not applicable; AOSD: adult-onset Still's disease; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; csDMARDs: conventional synthetic disease-modifying anti-rheumatic drugs; TNF: tumor necrosis factor; IL-6R: interleukin-6 receptor.
[a]Systemic activity score for AOSD, which is assessed using a modified Pouchot score.
[b]DAS28, disease activity score for 28-joints in RA patients.
[c]SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.
*p<0.05, **p<0.005, ***p<0.001, *vs*. RA; †p<0.005, ††p<0.001 *vs*. SLE.

Results

Characteristics of AOSD patients

Of the 56 patients with active AOSD, fever (≥39 °C), evanescent rash, arthralgia or myalgia, sore throat, liver dysfunction, and lymphadenopathy were noted in 54 (96.4%), 50 (89.3%), 46 (82.1%), 34 (60.7%), 30 (53.6%), and 24 (42.9%) patients respectively. Forty patients (71.4%) were classified as systemic subtype and 16 (28.6%) as chronic articular subtype. As illustrated in Table I, RA patients were significantly older than AOSD or SLE patients. A shorter duration of disease was observed in AOSD patients compared with RA patients or SLE patients. There were no significant differences in the age at entry or in female proportion between AOSD patients and HC (mean ± standard deviation, 38.6±11.9 *vs*. 38.3±8.9 years; 80.4% *vs*. 75.0%; respectively).

LncRNA expression signature in AOSD patients

Multiple regression analysis using *MIAT, THRIL, NTT, RMRP, PACERR,* and *NEAT1* expression values (ΔCT) as variables revealed that the levels of *MIAT, THRIL,* and *RMRP* could differentiate AOSD patients from healthy controls: *MIAT* (Odds ratio [OR] 0.17, 95% confidence interval [CI] 0.07–0.41, p<0.0005), *THRIL* (OR 2.94, 95%CI 1.44–6.02, p<0.005), and *RMRP* (OR 1.73, 95%CI 1.12–2.69, p<0.05). The expression levels of *MIAT, THRIL,* and *RMRP* were projected onto a 3D scatterplot using R software. As shown in Figure 1A, AOSD samples could be separated from control samples by the expression signature of these three lncRNAs.

Furthermore, 80% of the samples were randomly selected as the test dataset, and the other 20% of samples as the validation dataset to build a confusion matrix for evaluating the performance of classification algorithm using this new set. The accuracies of test dataset and validation dataset for diagnosing AOSD were 80.9% and 85.0%, respectively.

Establishment of AOSD prediction scores using lncRNA expression levels

To build up a lncRNA expression-based diagnostic model for AOSD, the regression coefficient was used as weight for each lncRNA expression ΔCT value in the scoring system: AOSD prediction score = (-1.76**MIAT*ΔCT)+ 1.08**THRIL*ΔCT+0.55**RMRP* ΔCT. The ROC analysis of our score for diagnosing AOSD showed AUC of 0.836 (p<0.001) at the cut-off score of 1.385, with sensitivity of 73.21% and specificity of 81.25% (Fig. 1B). Moreover, to investigate whether the lncRNA expression signature is specific for AOSD, we additionally enrolled 20 SLE and 20 RA patients to investigate lncRNA expression levels in their PBMCs. As shown in Figure 2A, this prediction score was significantly higher in AOSD compared with the scores in SLE or RA. Since sepsis is another differential diagnosis for AOSD, we downloaded a sepsis transcriptome cohort E-GEOD-32707 from ArrayExpress. By using multiple regression analysis, we found that the expression level of *MIAT, THRIL*, or *RMRP* could not be used to separate sepsis patients from healthy controls (OR 1.39, 95%CI 0.76-2.53, p=0.289; OR 1.08, 95%CI

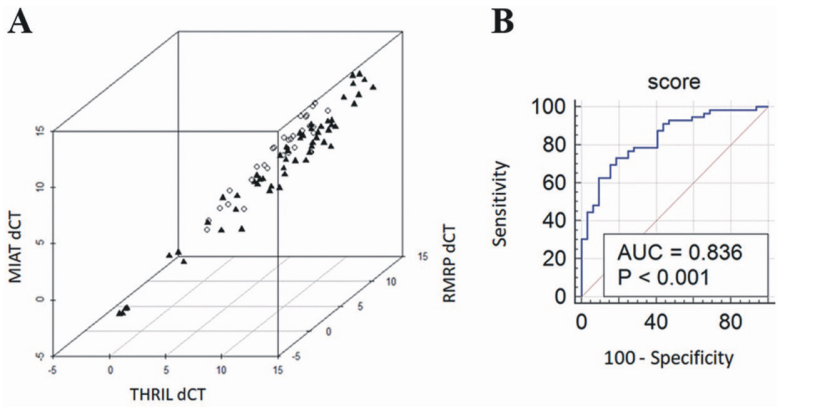

Fig. 1. Differentiation of AOSD patients from healthy controls by the expression signature of *LncRNAs*.
3D-scatterplot demonstrating *MIAT, THRIL* and *RMRP* delta CT (dCT) levels of each sample.
Circle: healthy controls (n= 32); triangle: AOSD patients (n= 56) (**A**). Receiver operating characteristic (ROC) curve analysis of the AOSD prediction score derived from *MIAT, THRIL* and *RMRP* expression levels on predicting the diagnosis of AOSD (**B**). Area under the curve (AUC)=0.836 at the cutoff score >1.385, with sensitivity of 73.21% and specificity of 81.25%.

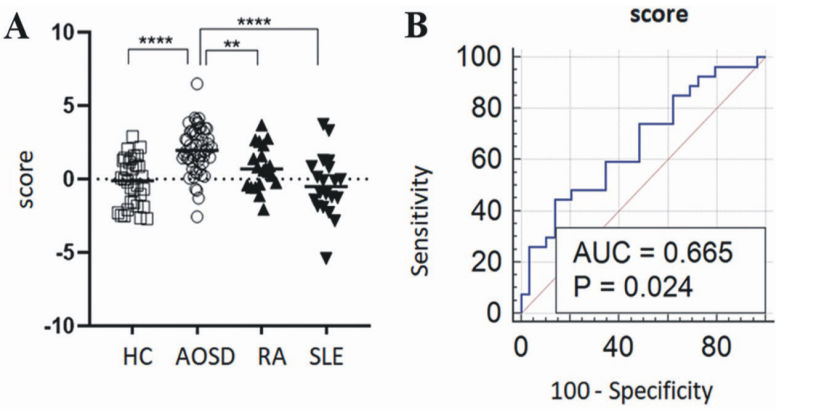

Fig. 2. AOSD prediction score in AOSD, RA, SLE, and HC, and for predicting the need of biological therapy in AOSD.
The differences in prediction scores among AOSD, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and healthy control (HC).
Lines represent medians.
***p* <0.01, *****p*<0.0001 calculated by Mann-Whitney U tests (comparing AOSD *vs*. HC, AOSD *vs*. RA, AOSD *vs*. SLE.
(**A**) Receiver operating characteristic (ROC) curve analysis of the AOSD prediction score derived from *MIAT, THRIL* and *RMRP* expression levels for predicting the need of biologic therapy in AOSD patients. (**B**) Area under the curve (AUC)=0.665 at the cutoff score >3.05, with sensitivity of 44.44% and specificity of 86.21%.

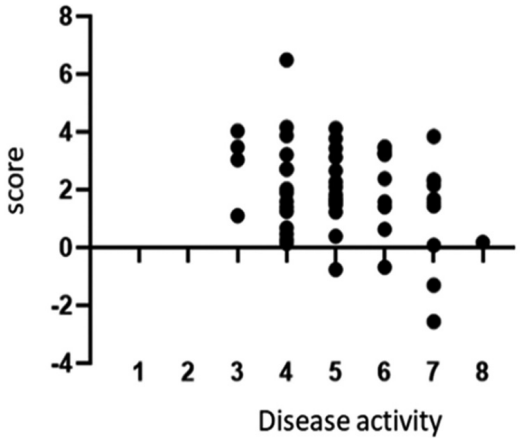

Fig. 3. Correlation between prediction score and systemic activity of AOSD.
The correlation coefficience between AOSD prediction score and systemic activity was determined by Pearson correlation test. A subgroup of AOSD samples with low AOSD prediction (lncRNA) scores had high disease activity scores. Pearson r = -0.28, *p*=0.035.

0.14–8.56, *p*=0.940; OR 0.15, 95%CI 0.00300, *p*=0.627, respectively).
To further evaluate the potential value of our scoring system on AOSD disease prognostication, we divided the patient group into those who needed biologic treatment in the disease course, and those who did not need biologics. Based on the baseline (before treatment) expression pattern of *MIAT, THRIL*, and *RMRP,* our score could differentiate ASOD patients who needed biologics from those who were stabilised by using conventional disease modifying anti-rheumatic drugs, with an AUC of 0.665, *p*=0.024 (Fig. 2B).

Association of AOSD prediction score with disease activity

We also analysed the correlation between the prediction score and AOSD disease activity, which was defined according to a modified Pouchot score described by Rau *et al*. (30). As shown in Fig. 3, a subgroup of AOSD samples with low AOSD prediction (lncRNA) scores had high disease activity scores, Pearson r=-0.28, *p*=0.03.

Change of lncRNA expression pattern after treatment in AOSD patients

To investigate whether the dysregulated lncRNA expression signature changes after treatment, we analysed the 6 lncRNA expression levels in 10 AOSD patients with available samples before and after 6-12 months of therapy. In these 10 patients, 4 were treated with prednisolone/cyclosporin, and 6 were treated with tocilizumab (anti-IL-6 receptor). Heterogenous changes in each lncRNA expression were observed in subjects after treatment. However, when analysing the whole lncRNA expression pattern, we found a strongly positive correlation between *NEAT1* expression ΔCT value and the levels of *MIAT, THRIL* and *RMRP* in treated AOSD patients, which was not observed in untreated individuals (Fig. 4).
In order to evaluate the potential interactions of lncRNAs contributing to the dysregulation in AOSD, we performed network analysis of proteins previously reported to be downstream of *MIAT, THRIL, RMRP* and *NEAT1* (27, 35-42) using STRING version 11.0 (https://

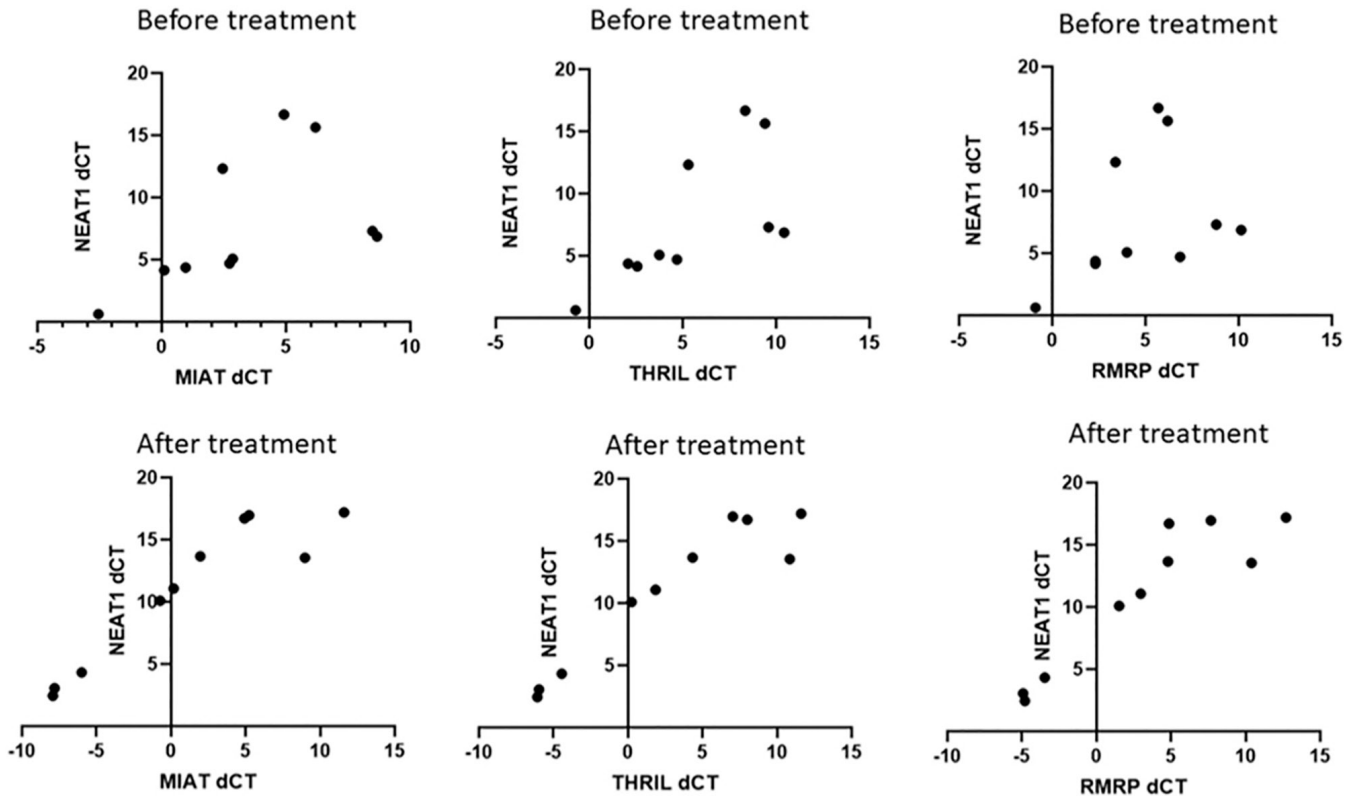

Fig. 4. Correlation between *NEAT1* expression ΔCT value and the levels of *MIAT, THRIL* and *RMRP* in AOSD patients before and after therapy. Strongly positive correlations were observed between *NEAT1* expression ΔCT (delta CT/dCT) value and the levels of *MIAT, THRIL* and *RMRP* in treated AOSD patients. Before treatment, Pearson r and *p*-values are as follows: *NEAT1* dCT *vs*. *MIAT* dCT, r=0.52, *p*=0.122; *NEAT1* dCT *vs*. *THRIL* dCT, r=0.68, *p*=0.030; *NEAT1* dCT *vs*. *RMRP* dCT, r=0.38, *p*=0.284. After treatment, Pearson r and *p*-values are as follows: *NEAT1* dCT *vs*. *MIAT* dCT, r=0.93, *p*=0.0001; *NEAT1* dCT *vs*. *THRIL* dCT, r=0.94, *p*<0.0001; *NEAT1* dCT *vs*. *RMRP* dCT, r=0.92, *p*=0.0002.

string-db.org/cgi/network.pl). Known and predicted interactions among these downstream molecules were noted, with enrichment in pathways involving I-kappaB kinase/NF-kappaB signalling (GO:0007249), positive regulation of interleukin-6 signalling (GO:0032755), activation of MAPK activity (GO: 0000187), phosphatidylinositol-3 kinase signalling (GO: 0014065), and T helper cell lineage commitment (GO: 0002295) (Fig. 5).

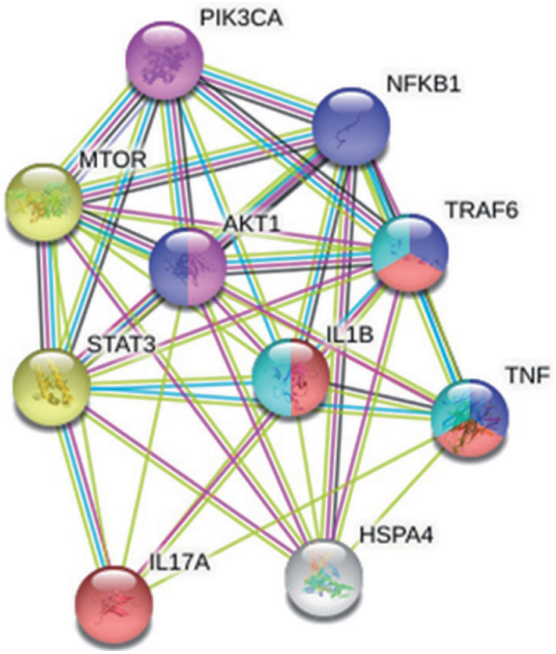

Fig. 5. Network analysis of interactions of lncRNA-related molecules using STRING (Protein-Protein Interaction Networks Functional Enrichment Analysis). Network analysis was performed on the potential interactions of proteins previously reported to be downstream of *MIAT, THRIL, RMRP* and *NEAT1* using STRING version 11.0 (https://string-db.org/cgi/network.pl). Colours represent molecules enriched in specific pathways: red, GO:0032755, positive regulation of interleukin-6 production; blue, GO:0007249, I-kappaB kinase/ NF-kappa B signaling; light blue, GO:0000187, activation of MAPK activity; yellow, GO:0002295, T-helper cell lineage commitment; pink, GO:0014065, phosphatidylinositol 3-kinase signaling.

LncRNA expression patterns in human monocytic cell line and primary human cells stimulated with TLR ligands

Since TLR3 activation has been reported to be involved in AOSD pathogenesis [10], we examined the lncRNA expression patterns upon TLR3 ligand poly(I:C) or TLR4 ligand LPS stimulations, and revealed the different lncRNA expression signatures (Fig. 5). We observed increased *MIAT* and decreased *THRIL* levels in THP-1 cells stimulated with poly(I:C), which was similar to the change of lncRNA expression patterns in AOSD patients shown in our previous 3D-scatterplot. In LPS-treated THP-1 cells, however, *MIAT* downregulation combined with upregulations of *NEAT1* and *RMRP* was detected at 4 hours after stimulation (Fig. 6). In addition,

poly(I:C) upregulated *NTT* expression already at 4 hours of stimulation; while LPS increased *NTT* expression levels after 24 hours of stimulation (Fig. 6).

In order to investigate whether the results observed in THP-1 cell line can also be applied to primary human cells, we further performed poly(I:C) and LPS stimulation experiments on lymphocytes and monocytes derived from healthy control PBMCs using overnight adhesion method (Supplementary Fig 1A-H). Similar to those observed in THP-1 cells, both poly(I:C) treated lymphocytes and monocytes showed trend of elevated *MIAT* and decreased *THRIL* at 24 hours of stimulation; and the changes were most prominent in monocytes (Suppl. Fig 1C-D). In contrast to THP-1 cells, 4-hour LPS-treated lymphocytes and monocytes had increased *MIAT* expression (Suppl. Fig. 1E-F). Furthermore, 4-hour LPS stimulated monocytes showed upregulation of both *RMRP* and *THRIL* (Suppl. Fig 1F). After 24 hours of LPS treatment, *MIAT* upregulation was still observed in the two cell subsets, while *THRIL* and *NTT* upregulations were more prominent in lymphocytes (Suppl. Fig. 1G-H).

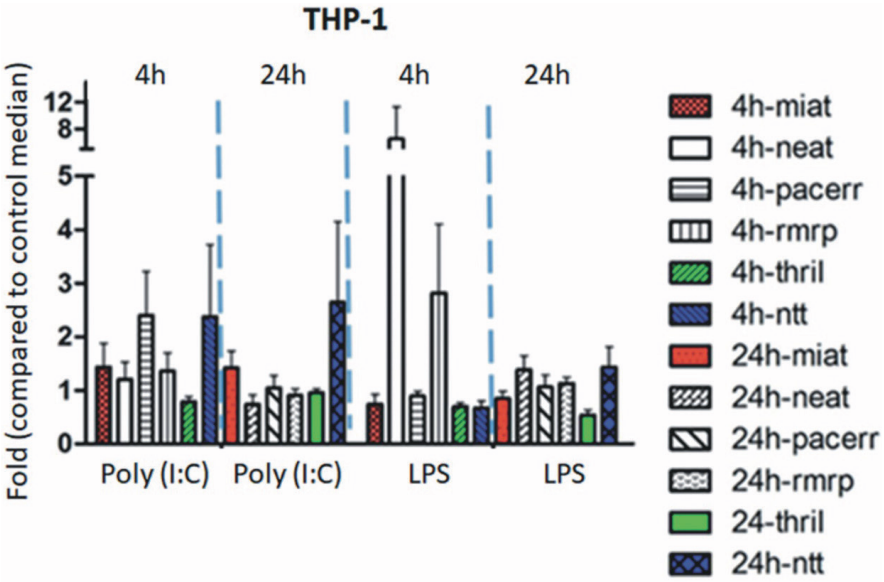

Fig. 6. LncRNAs expression patterns upon poly(I:C) or LPS stimulation in human monocytic cell line THP-1.

THP-1 cells were stimulated with 50μg/ml poly(I:C) or 100 ng/ml LPS for 4 hours or 24 hours. THP-1 cells cultured in LPS-free RPMI medium only for 4 hours and 24 hours were used as controls for the respective experiments. The expressions of lncRNAs were measured by real-time PCR. The 4h poly(I:C) stimulation: n=6~8; 4h LPS stimulation: n=3~5; 24h poly(I:C) stimulation: n=5~6; 24h LPS stimulation: n=3~5. Bars represent mean ± SEM. The fold of expression of lncRNAs in THP-1 cells analysed was calculated by 2 ^ -ΔΔCT [(CT^{lncRNA} – CT^{GAPDH}) - median ΔCT of control replicates].

Discussion

Given clinical heterogeneity and lack of specific biomarkers (30, 43), AOSD diagnosis is usually delayed. LncRNAs have been reported to be key regulators of immune responses (14-17), and the downstream cytokines play pathogenic roles in AOSD (3-6). In this study, we developed an AOSD diagnostic scoring system based on the expression signature of lncRNAs *MIAT, THRIL* and *RMRP*. AOSD patients were found to have simultaneously decreased *MIAT* ΔCT and increased *THRIL* and *RMRP* ΔCT values, which contribute to higher prediction scores for AOSD compared with the scores for SLE or RA. At the cut-off score value of >1.385, our score showed acceptable sensitivity (73.21%) and specificity (81.25%) on diagnosing AOSD (AUC=0.836). Furthermore, we demonstrated that the combined expression signature of *MIAT, THRIL* and *RMRP* could differentiate AOSD from SLE, RA and potentially also sepsis. Since lncRNA expression has been reported to be upstream of various immune responses (14-17, 26), we are interested in studying if our scoring system could provide early AOSD diagnosis in further investigations.

In the aspect of AOSD prognostication, our score showed a good specificity (86.21%) at the cut-off score of >3.05 for identifying patients requiring the use of biologics, despite a low sensitivity (44.44%). Unlike the expression of microRNA-134 (miR-134) and frequency of circulating T helper type 17 (Th17) cells, which were reported to have strong positive correlation with AOSD disease activity score, our AOSD prediction score showed a mildly negative correlation with disease activity (Pearson r= -0.28, p=0.03) (3, 10). It has been shown that miR-134 expression and percentages of Th17 cells in blood also positively correlated with the levels of known pathogenic cytokines in AOSD patients, especially interleukin-18 (IL-18), a cytokine found to be involved in disease activity (3, 10, 44). In contrast, lncRNAs are known to play regulatory and fine-tuning roles in immune responses (26, 45). Therefore, the observed lncRNA expression signature could be the result of intricate interactions of different pathways in AOSD pathogenesis, instead of the impact of single cytokine dysregulation. This might partly explain why the lncRNA expression pattern specifically observed in AOSD only mildly correlated with AOSD disease activity.

Because of the reported importance of IL-18 in AOSD, we further evaluated the ROC curves of IL-18 on AOSD diagnosis and prognostication using our cohort. Compared with our lncRNA score, IL-18 alone had higher sensitivity (82.5%) and specificity (94.74%) on diagnosing AOSD at the optimal cut-off value. However, IL-18 alone could not predict the use of biologics in AOSD treatment (AUC=0.568, p=0.48 at optimal cut-off). Recently, Sharif *et al.* reveal that ferritin takes a pathogenic role in inflammation, which further stimulates secretion of proinflammatory cytokines (46). AOSD is characterised by macrophage activation with elevation of circulating ferritin (47). Given a common finding of hyperferritinaemia in AOSD, coronavirus disease 2019 (COVID-19), anti-melanoma differentiation-associated protein 5 (MDA5)-positive dermatomyositis, and macrophage activation syndrome

(48-49), the specificity for diagnosing AOSD is relatively low.

It has been reported that lncRNAs *MIAT, THRIL* and *RMRP* could modulate several common pathways important in immune responses (35, 36, 38, 39, 50). *MIAT* has been shown to exert positive regulatory functions on PI3K/Akt signalling in atherosclerosis and melanoma (35, 51); while suppressive effect of *MIAT* on TNF-α and IL-1ß was found in a model of macrophage inflammation (36). *THRIL* is reported to activate PI3K/Akt signalling in inflammatory response of RA synoviocytes and autophagy of endothelial progenitor cells (37, 50). Contrarily to *MIAT, THRIL* was found to upregulate TNF-α in different conditions (38, 52). *RMRP* has also been reported to modulate PI3K/Akt/mTOR pathway (39, 53), and Th17 effector cell functions (27). Moharamoghli *et al*. had detected an increase of both *RMRP* and IL-17 levels in patients of RA; however, no correlation was observed between *RMRP* and IL-17 (54). Taken together, our finding on the AOSD-distinctive lncRNA expression signature of *MIAT, THRIL* and *RMRP* suggests possible dysregulations in PI3K/Akt, TNF-α, and IL-17 pathways.

Interestingly, *NEAT1* expression level was found to be strongly correlated with the expression of *MIAT, THRIL* and *RMRP* in 10 AOSD patients after treatment (4 were treated with prednisolone/cyclosporin, and 6 treated with IL-6R inhibitor). This phenomenon was not observed in PBMCs of the 10 AOSD patients before treatment. *NEAT1* has been reported to activate PI3K/Akt/mTOR signalling, and has been shown to regulate TRAF6/NF-κB and the secretion of related cytokines, such as TNF-α and IL-6 (55-57). Whether the correlation of expression pattern between *NEAT1* and other lncRNAs could be a sign of recovery from dysregulations in PI3K/Akt signalling, TNF-α secretion, and T cell activation in treated AOSD patients requires further investigations. Our study is limited that we only had followed-up blood drawn from 10 AOSD patients, and 6 of them had used anti-IL-6 receptor antibody, a biologic possibly affecting the IL-6 and other lncRNA-regulated immune pathways. A larger cohort with more AOSD patients treated without biologics should be recruited for further analysis.

Our finding of increased *MIAT* expression and decreased *THRIL* expression in AOSD patients as compared with healthy controls resonates with the hypothesis that AOSD pathogenesis might involve TLR3 activation (10) and our previous results (21) that *MIAT* levels could be enhanced in THP-1 cells treated with TLR3 ligand poly(I:C). Using THP-1 and primary monocytes as cell models, we showed that the change of lncRNA expression patterns after 24h poly (I:C) stimulation was similar to those observed in our AOSD patients, further supporting their potential roles in disease pathogenesis. However, our cell model could not totally explain the mechanisms leading to the dysregulations of lncRNAs in AOSD.

Furthermore, disease course of AOSD may vary considerably (31, 32) and can be divided into systemic and articular subtypes. This study is also limited in that due to the relatively small AOSD cohort size, we did not separate AOSD samples into systemic and articular subtypes. Several investigators including us have previously revealed significantly higher TNF-α levels in patients with AOSD-articular subtype (4, 5), for whom TNF-α inhibitors have been proved effective (6). As mentioned in previous paragraphs, *MIAT* and *THRIL* are regulators for TNF-α secretion. It is possible that the lncRNA expression signature differs between the two AOSD subtypes.

In conclusion, we provide an AOSD diagnostic scoring system based on the expression signature of *MIAT, THRIL* and *RMRP*. Our findings might aid in early AOSD diagnosis and decisions in choosing optimal therapeutic agents, the so-called personal medicine. Further investigations are needed to uncover the mechanisms of lncRNA dysregulation in AOSD.

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