Global microRNA profiling of peripheral blood mononuclear cells in patients with Behçet's disease

G.L. Erre[1], M. Piga[2], C. Carru[3], A. Angius[4,5], L. Carcangiu[4], M. Piras[1], S. Sotgia[3], A. Zinellu[3], A. Mathieu[2], G. Passiu[1], M. Pescatori[6]

[1]*Cattedra e Unità di Reumatologia, Dipartimento di Medicina Clinica e Sperimentale, Università e AOU di Sassari, Sassari, Italy;*
[2]*Cattedra e Unità di Reumatologia, Dipartimento di Scienze Mediche, Università e AOU di Cagliari, Monserrato, Italy;*
[3]*Dipartimento di Scienze Biomediche, Università di Sassari, Sassari, Italy;*
[4]*Istituto di Ricerca Genetica e Biomedica (IRGB), CNR, Monserrato, Italy;*
[5]*CRS4, Parco Tecnologico della Sardegna, Pula, Italy;*[6]*Health e-solutions, Rotterdam, The Netherlands.*

Gian Luca Erre, MD, PhD
Matteo Piga, MD
Ciriaco Carru, Prof.
Andrea Angius, PhD
Laura Carcangiu, PhD
Marco Piras, MD
Salvatore Sotgia, MSc
Angelo Zinellu, PhD
Alessandro Mathieu, Prof.
Giuseppe Passiu, Prof.
Mario Pescatori, MD

Please address correspondence to:
Gian Luca Erre, MD, PhD,
UOC Reumatologia,
Dipartimento di Medicina Clinica e Sperimentale,
AOU e Università degli Studi di Sassari
Viale San Pietro 8,
07100 Sassari, Italy
E-mail: e.gianluca@libero.it

Received on October 26, 2014; accepted in revised form on May 27, 2015.

Clin Exp Rheumatol 2015; 33 (Suppl. 94): S72-S79.

Key words: microRNA, Behçet, innate immunity, Toll-like receptor, T-cell receptor, miR-199, miR-720, biomarkers

Competing interests: none declared,

ABSTRACT

Objective. *To explore the post-transcriptional regulation of the peripheral blood mononuclear cells (PBMCs) transcriptome by microRNAs in Behçet's disease (BD).*

Methods. *Using TaqMan Low Density Array-based microRNAs expression profiling, the expression of 750 mature human microRNAs in PBMCs from 5 BD patients and 3 healthy controls (HC) was compared. The expression of deregulated microRNAs was then validated by quantitative real time-polymerase chain reaction (qRT-PCR), in 42 BD patients and 8 HC.*

Results. *In the initial screening, 13 microRNAs appeared deregulated in BD vs HC. Among them, the differential expression of miR-720 and miR-139-3p was confirmed by qRT-PCR, ($p<0.05$ and FDR <5%). Areas under the receiver operating characteristic curve for miR-139-3p, miR-720 and miR-139-3p + miR-720 in the validation cohort were 0.84, 0.87 and 0.92 respectively, indicating good discrimination between BD patients and HC. Post-hoc analysis showed that 9 out of 13 microRNAs from the discovery phase were significantly upregulated in active vs. quiescent BD, suggesting inflammation as a key regulator of microRNAs machinery in BD.* In silico *analysis revealed that several BD candidate susceptibility genes are predicted target of significantly deregulated microRNAs in active BD. A significant enrichment in microRNAs targeting elements of the Toll-like receptor (TLR) and T-cell receptor signalling pathways was also assumed.*

Conclusion. *miR199-3p and miR720 deserve further confirmation as biomarkers of BD in larger studies. PBMCs from active BD displayed a unique signature of microRNAs which may be implicated in regulation of innate immunity activation and T-cell function.*

Introduction

Behçet's disease (BD) is a systemic inflammatory disease mainly characterised by recurrent orogenital aphthous ulcers, uveitis, and skin inflammation (1). The aetiology of BD is still elusive but it is believed that the disease is the result of the interaction between environmental factors and genetic predisposition. In fact, the distinctive geo-epidemiology of BD (2) and its close association with HLA-B51 side by side with familial clustering and high concordance in monozygotic twins all strongly account for a relevant pathogenetic contribution of the genetic background (3). A key component of genetic susceptibility to BD is located on chromosome 6 within the MHC region, being primarily related to HLA-B*51:01 and to other HLA (HLA-C, HLA-DR) and HLA-related genes (tumour necrosis factor alpha [TNF-alpha] and MICA*009 [MHC class I chain-related gene A] polymorphisms) (4). In addition, genome-wide association studies (GWAS) in different ethnical cohorts have identified several susceptibility loci for BD including, IL23R-IL12RB2, IL-10, Endoplasmic Reticulum Aminopeptidase 1 (ERAP1), signal transducer and activator of transcription 4 (STAT4), Toll-like receptor (TLR) and C-C chemokine receptor type 1 (CCR1) (5-10). Although these studies help to clarify the genetic basis of BD, functional data linking susceptibility genes to BD pathogenesis are still lacking.

microRNAs are small (19–24 nucleotide) noncoding RNAs, which affect the regulation of gene expression by either inhibiting translation or promoting degradation of specific mRNA transcripts. More than 900 microRNAs have been identified in mammals. MicroRNAs have long been known for their role in cellular proliferation, organ development, homeostasis and function. More-

over, microRNAs play a crucial role in both innate and adaptive immunity, including the control of differentiation and function of various immune cell subsets. As a consequence, a growing body of evidence has demonstrated the implication of specific microRNAs in the pathogenesis of a variety of autoimmune and inflammatory conditions (11).

From an epidemiological point of view, data about microRNAs expression in BD in Sardinia could be of particular interest. The Sardinian population is characterised by a homogeneous genetic background with little gene inflow and a peculiar high frequency of autoimmune diseases (*e.g*, type 1 diabetes and multiple sclerosis) (24, 25). Lower background genetic variation resulting in reduced background variation in microRNAs expression could contribute to maximise the chances of identifying *true* positive associations. Furthermore, from a pathophysiological perspective, it is likely that Sardinian BD patients share more homogeneous genetic traits (25) whose identification may suggests a defined pathogenetic mechanism, but with a less universal significance than that detectable in non-isolated populations, since it is determined by the interaction of a lower number of gene variants with a reduced number of environmental variables.

Significantly increased frequencies of the miR-196a2/rs11614913 TT genotype and T allele and polymorphism of miR-146a have been reported to be associated to BD (12, 13). Zhou *et al*. (14) found a significant lower expression of miR-155 in BD patients with active uveitis, they validated experimentally TAK1-binding protein 2 (TAB2) as a target of miR-155 and supposed that miR-155 would be able to suppress proinflammatory cytokines production via TLR/IL-10 signaling cascade by targeting TAB2. Afterwards, it has been proposed that downregulated miR-23b expression in $CD4^+$T cells from active BD patients relieves negative control of Notch-1 (a type 1 transmembrane protein family member) signalling leading to expansion of Th17 cells (15). To the best of our knowledge global microRNAs profiling in BD has not been performed until now. To address this, we explored microRNAs expression profile through microarray analysis and qRT-PCR in BD PBMCs as compared to their counterpart from healthy controls (HC).

Material and methods

Patients

Consecutive unrelated Sardinian BD patients diagnosed according to the International Study Group (ISG) criteria (16), referred to the Rheumatology Units of Sassari and Cagliari between April 2012 and January 2013 were enrolled in the study. HC were recruited on a voluntary bases and were enrolled after a careful examination aimed at rule out signs and symptoms of inflammation. Patients and controls originated from various areas of Sardinia and were representative of the population distribution.

For each patient enrolled in the study data regarding disease duration, clinical features and treatment were systematically registered. Results of laboratory examinations, including counts of white blood cells, erythrocyte sedimentation rate and C-reactive protein, were registered. When available, previously determined HLA-B51 and HLAB*51:01 positivity was registered. BD patients were stratified according with clinical manifestation in 5 subsets: mucocutaneous BD, ocular BD, neurological BD, vascular BD, intestinal BD. At the time of blood sampling, BD patients with at least one symptom/lesion in the previous 2 weeks (including oral and/or genital aphthous ulcers, specific skin lesions, uveitis, vascular lesions, arthritis, gastrointestinal lesions, central and peripheral nervous system lesions, and pulmonary involvement) were classified to have "active BD"; patients not fulfilling these criteria were classified as having "quiescent BD".

Experiments

All the experiments were performed in the CRS4 Sequencing and Genotyping Platform Centre in Pula (Sardinia, Italy) with the exception of RNA isolation from PBMCs of subjects enrolled in the discovery phase which was performed in the Laboratory of Clinical Biochemistry of the University of Sassari. Blood samples were collected in EDTA using vacuum tubes and PBMCs were separated by Ficoll-Hypaque density-gradient centrifugation. Total RNA was isolated by TRIZOL standard method. RNA quantity and purity was evaluated by spectrophotometric analysis using a Nanodrop ND-1000 apparatus (Thermo Fisher Scientific, Wilmington, USA) and the Agilent Bioanalyzer 2100 and the RNA 6000 Nano kit (Agilent technologies, Santa Clara, CA, USA). RNA quality was rated according the RNA integrity number (RIN): an algorithm that assigns an integrity number to an eukaryote total RNA sample.

Discovery phase

Profiling of human miRnome in our discovery phase cohort (5 BD and 3 HC, PBMC) was performed using the Human MicroRNA TaqMan Low Density Array (TLDA, TaqMan Array Human MicroRNA A+B Cards Set v3, Applied Biosystems) using an ABI Prism 7900HT. This platform allows the analysis of 754 different human microRNAs. The expression level of the small nuclear RNA RNU44 was used as normalisation control. Log2 expression levels were calculated using the N-ΔCt transformation, where N can be any number, generally greater than the maximum ΔCt value in the dataset. Log2 expression measures were imported in BRB ArrayTools and analysed using the class comparison function. miRs passing the statistical threshold, were tabulated along with FDR estimate, mean expression in each class, and fold change.

Validation phase

The differential expression of microRNAs significantly deregulated at the microarray-based screening was validated in a independent large set of patients and controls, constituting our validation cohort. Further informations on clinical and demographic characteristics of the validation cohort are described in Table I. To this aim, a custom TLDA card was designed to include all the essays for the microRNAs passing the statistical threshold in the discovery phase (Applied Biosystems, Foster City, California). In addition to the candidate microRNAs identi-

fied in the microarray screening, we also included in the TLDA card a set of 10 microRNAs selected *a priori*, on the basis of their *in silico* predicted, or experimentally demonstrated ability to regulate genes and pathways involved in the pathogenesis of BD. An additional pfd file shows this in more detail [see Additional file 1].

The experiments were conducted according to manufacturer instructions, using a 7900 real-time PCR system (Applied Biosystems).

Data analyses

A primary data analysis was performed using the Sequence Detection System (SDS) software (v. 2.4, Applied Biosystems, Grand Island, NY). The expression level of the small nuclear RNA RNU44 was used as normalisation control. Log2 expression levels were calculated using the N-ΔCt transformation, where N can be any number, generally greater than the maximum ΔCt value in the dataset. Log2 expression values were imported in BRB ArrayTools, or other analysis software. Secondary data analysis, including clustering and prediction analysis, was performed in BRB ArrayTools (developed by Dr. Richard Simon and BRB-ArrayTools Development Team), SPSS (release 20.0, Chicago,
IL, USA) or Graphpad (Prism, California, USA). microRNAs differentially expressed between active and quiescent BD patients were clustered and displayed as heatmap using the clustering tool as implemented in BRB ArrayTools.

Receiver operating characteristic (ROC) analysis

We evaluated the performance in differentiating BD patients from HC for each differentially expressed miR in the validation phase by means of sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), receiver operating characteristic (ROC) curves and area under the curve (AUC) with corresponding 95% confidence intervals. As optimal cut-off scores were selected those yielding the highest sum of specificity and sensitivity. To evaluate whether a combination of microRNAs perform better than any of the single we obtained a ROC analysis fitting as a single variable the predicted probability. Predicted probability was obtained fitting the condition (BD) as the dependent variable and multiple candidate microRNAs as the covariates and running a binomial logistic regression analysis.

ROC curves were constructed and AUC were estimated by SPSS software (release 20.0, Chicago, IL, USA). A *p*-value <0.05 was considered statistically significant.

Target identification and pathway analysis

We examined biological relevance of significantly deregulated microRNAs performing an "educated" search for

Table I. Demographic, clinical and laboratory features of discovery and validation cohorts of BD patients.

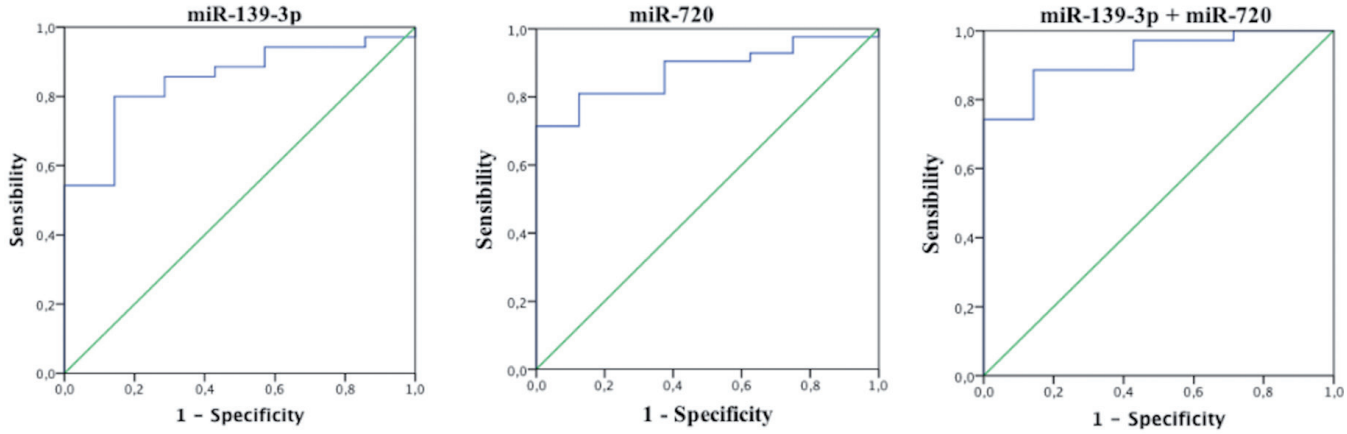
	HC n=11	BD n= 47	BD discovery cohort, n=5	BD validation cohort, n=42	*p* discovery *vs*. validation cohort
Age, yrs	39 (28-54)	43 (23-69)	40 (30-49)	46 (23-69)	0.19
Female sex, n (%)	8 (72.7%)	35 (74.5%)	5 (100%)	30 (71.4%)	0.16
BD disease duration, years		14 (2-33)	10 (2-15)	14 (2-33)	0.2
Active disease, n (%)		19 (40.4%)	1 (20%)	18 (42.9%)	0.32
Mucocutaneous BD, n (%)		18 (38.3%)	2 (40%)	16 (38%)	0.93
Ocular BD, n(%)		19 (40.4%)	3 (60%)	16 (38%)	0.34
Neuro BD, n (%)		4 (8.5%)	0	4 (9.5%)	0.47
Angio BD, n (%)		3 (6.4%)	0	3 (7.1%)	0.53
Intestinal BD, n (%)		3 (6.4%)	0	3 (7.1%)	0.53
NSAIDs use, n (%)		4 (8.5%)	1 (20%)	3 (7.1%)	0.33
Low steroids use, n (%)		29 (61.7%)	3 (60%)	26 (61.9%)	0.93
Medium/high steroids use, n (%)		6 (12.8%)	1 (20%)	5 (11.9%)	0.6
DMARDs use, n (%)		32 (68.1%)	3 (60%)	29 (69%)	0.68
Colchicine use, n (%)		10 (21.3%)	2 (40%)	8 (19%)	0.27
Cyclophosphamide use, n (%)		1 (2.1%)	0	1 (2.4%)	0.72
Anakinra use, n (%)		1 (2.1%)	0	1 (2.4%)	0.72
AntiTNF use, n (%)		1 (2.1%)	0	1 (2.4%)	0.72

Continuous non normally distributed data are expressed as median (range) and comparison was made using Mann-Whitney U test. Normally distributed data are expressed as mean ± 1 SD and compared by t-test. Proportions were compared by chi-quadro.
ns: not significant; NSAIDs: non steroidal anti-inflammatory drugs; Low steroids: <10 mg/day prednisone equivalent; Medium-high steroids: ≥10mg/dayDMARDs: disease modifying anti-rheumatic drugs; Anti-TNF: anti-Tumour necrosis factor agents.

Table II. miR differentially expressed in BD PBMCs vs HC PBMCs.

miR Unique ID	FDR	Style	Fold change	*p*-value
Discovery phase				
hsa-miR-200b-002251	/	Down	0.27	0.003
hsa-miR-571-001613	/	Up	2.81	0.006
hsa-miR-127-000452	/	Up	3.12	0.007
hsa-miR-224-002099	/	Down	0.27	0.008
hsa-miR-98-000577	/	Down	0.55	0.013
hsa-miR-573-001615	/	Up	1.09	0.016
hsa-miR-770-5p-002002	/	Up	5.69	0.016
hsa-miR-199a-3p-002304	/	Down	0.61	0.025
hsa-miR-370-002275	/	Up	5.01	0.033
hsa-miR-139-3p-002313	/	Up	6.012	0.034
hsa-miR-520c-3p-002400	/	Down	0.0096	0.036
hsa-miR-720-002895	/	Up	1.98	0.042
hsa-miR-330-000544	/	Up	2.11	0.048
Validation phase				
hsa-miR-139-3p-002313	0.0419	Up	2.92	0.0032
hsa-miR-720-002895	0.0419	Up	2.4	0.0046

Differentially expressed miR were selected on the basis of a $p \leq 0.05$ in the discovery phase and on the basis of a $p \leq 0.05$ and FDR <5%. in the validation phase. Two (miR-139 and miR-720) of the 13 deregulated miR at the discovery phase were confirmed upregulated at the validation phase.



Test	Sensitivity	1-Specificity	AUC (95% CI)	PPV	NPV	TP	FP	TN	FN
miR-139-3p*	0.80	0.14	0.84 (0.71-0.98)	0.97	0.46	28	1	6	7
miR-720	0.81	0.12	0.87 (0.78-0.97)	9.97	0.47	34	1	7	8
miR-139 3p+ miR-720*	0.88	0.14	0.92 (0.83-1)	0.97	0.60	31	1	6	4

*missing data: n=8; UC: area under the curve, PPV: positive predictive value; NPV: negative predictive value; TP: true positive; FP: false positive; TN: true negative; FN: false negative.

Fig. 1. Receiver Operating Characteristic (ROC) curves for performance of miR139-3p, miR720 and combination of miR139-3p and miR-720.

their interaction with candidate susceptibility genes for BD (TNF-alpha, MICA, IL23R-IL12RB2, ERAP1, STAT4, TLR, CCR1), using TarBase v. 6.0 database for experimentally validated interactions (17, 18) and DIANA microT-CDS database for predicted interactions (19, 20).

An "expanded" target gene prediction analysis was performed using miRecords (21). Putative targets were selected when predicted by at least 4 prediction algorithms and conserved among species. In addition we manually browsed the PubMed database searching for context-relevant, experimentally validated microRNA-gene interactions. Direct microRNAs to Pathway analysis was performed using miRPath v. 2.0 (22, 23) using intersection of pathways targeted by selected microRNAs.

Statistic

Differences were statistically evaluated by Student's *t*-test. The Mann-Whitney U-test was used to compare non normally distributed data. Unless otherwise stated, $p \leq 0.05$ was considered to be statistically significant. In the discovery phase, differentially expressed miR were selected on the basis of a $p \leq 0.05$, in order to contain the effect of type-1 error. In the validation phase, a more restrictive analysis has been performed selecting significantly differentially regulated microRNAs on the basis of a false discovery rate (FDR) <5%. A per gene FDR estimate was computed in BRB ArrayTools using the Benjamini and Hochberg approach. All the statistical analysis was performed using SPSS or BRB ArrayTools.

Ethics

The study was approved by the Ethics Committees of ASL1 Sassari (1037/CE, 2012) and AOU of Cagliari (224/CE) and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Results

Patients and controls

We studied 47 BD patients, 34 referring to the Rheumatology Unit of Cagliari and 13 to the Rheumatology Unit of Sassari, (23–69 years of age [median 43 years], 35 female, with disease du-

Table III. miR differentially expressed in active BD PBMCs *vs*. quiescent BD *vs*. HC PBMCs.

miR Unique ID	Class 1 GMI Active BD	Class 2 GMI Quiescent BD	Class 3 GMI HC	Pairwise significant	FDR	*p*-value
hsa-miR-330-000544	290.8	102.85	121.08	(2,1) (3,1)	0.000796	0.0000534
hsa-miR-139-3p-002313	239.86	101.18	52.06	(2,1) (3,1)	0.000796	0.0001226
hsa-miR-199a-3p-002304	8372.19	2622.77	3620.57	(2,1)	0.000796	0.0001769
hsa-miR-720-002895	27516.33	14822.92	8034.54	(2,1) (3,1)	0.00125	0.0005548
hsa-miR-98-000577	301.29	118.96	125.98	(2,1) (3,1)	0.0014	0.0007674
hsa-miR-224-002099	137.36	37.76	48.81	(2,1)	0.00323	0.0019763
hsa-miR-200b-002251	100.33	45.72	40.88	(2,1) (3,1)	0.00502	0.0033474
hsa-miR-127-000452	968.84	403.63	288.09	(2,1) (3,1)	0.00557	0.0043359
hsa-miR-370-002275	226.11	92.41	102.14	(2,1) (3,1)	0.0521	0.0463496

Differentially expressed miR were selected on the basis of a $p \leq 0.05$ and FDR <5%. GM!: geometrical mean of intensities.

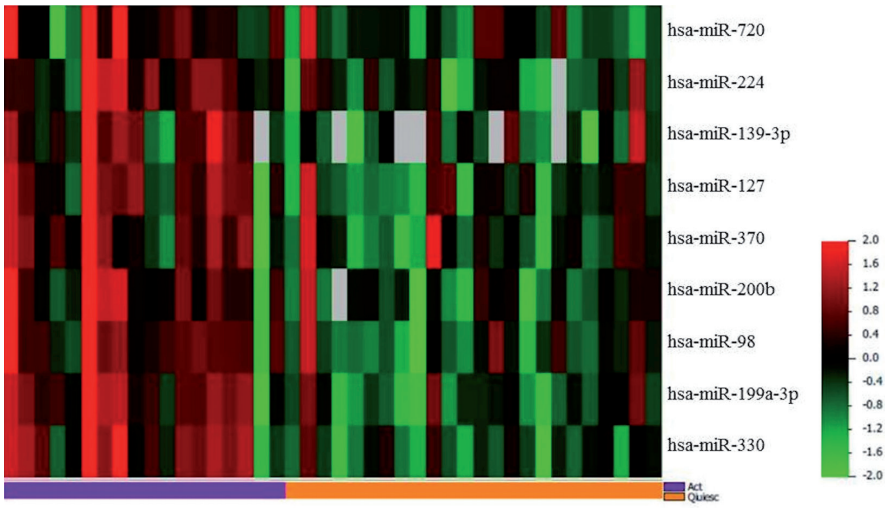


Fig. 2. microRNA expression heatmap active *vs*. quiescent BD.
Heatmap depicting expression profile of microRNAs in PBMCs from active BD (dark violet) and quiescent BD (dark orange).

Table IV. Susceptibility genes and deregulated miR validated and predicted interactions.

Candidate gene, ENSG, Chromosome location	Predicted miR	Validated miR
TNF, ENSG00000232810, 6p21	-	-
MICA, ENSG00000204520, 6p21	-	-
ERAP-1, ENSG00000164307, 5q15	Let-7f, miR-330	-
KIR (KIR3DL1), ENSG00000167633, 19q13.4	-	-
IL-10, ENSG00000136634, 1q31.32	miR-98, Let-7a, Let 7f	-
IL23R, ENSG00000162594, 1p31	-	-
IL12RB2, ENSG00000081985, 1p31	Let-7a	
IFNGamma, ENSG000001115374, 12 q15	-	-
NOS2, ENSG00000007171, 17q11.2	-	-
AGER, ENSG00000204305, 6p21	-	-
TLR4, ENSG00000136869, 9q33.1	miR-23, Let-7a, Let-7f,	
miR-200b, miR-98, miR-224	-	
TLR2, ENSG00000137462, 4q32	-	miR-146a
HMGB1, ENSG00000189403, 13q12.3	Let-7a, Let-7b, miR-200b,	
	miR-224	-
VEGF-A, ENSG00000112715, 6p12	-	miR-330, miR200b
CCR1, ENSG00000163823, 3p21	miR-330	-

Analysis has been performed using TarBase v6.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index) and miRecords http://mirecords.biolead.org.
ENSG Ensembl gene id, Chromosome location; TNF: tumour necrosis factor; MICA: MHC class I polypeptide-related sequence A; ERAP-1: Endoplasmic reticulum aminopeptidase 1; KIR: Killer cell immunoglobulin-like receptor; NOS2: Nitric oxide synthase 2; AGER: Advanced glicated endproduct receptor; TLR-4/2: Toll-like receptor 4/2; HMGB1: High Mobility Group Box 1; VEFG-A: vascular endotelial growth factor A; CCR1: C-C chemokine receptor type 1.

ration between 2 and 33 years [median 10 years] and 11 HC (28-54 years of age [median 39], 7 female). Five BD patients and 3 HC from the Rheumatology Units of Sassari were enrolled in the discovery phase as screening cohort; the remaining 42 patients and 8 HC were enrolled in the validation phase. Nineteen out of 42 (40.4%) patients had active disease at the moment of blood collection, according to protocol classification criteria. A large proportion of the BD sample presented with mucocutaneous and ocular manifestations (38.3% and 40.4% respectively). All BD patients were under treatment at the time of blood collection. Demographic and clinical characteristics of BD patients and HC are summarised in Table I.

Discovery phase

Of the 750 mature human microRNAs analysed, 13 resulted significantly modulated in PBMCs of BD patients as compared with PBMCs from HC: 8 were upregulated (miR-571, miR-127, miR-573, miR-770, miR-370, miR-139-3p, miR-720 and miR-330), whereas 5 were downregulated (miR-200b, miR-520c-3p, miR-199a-3p, miR-224 and miR-98) (Table II). However, unsupervised hierarchical clustering analysis of significantly deregulated microRNAs did not allow a clear separation of BD from HC (data not shown).

Validation phase

Taqman qRT-PCR analysis carried out on all the microRNAs scored as differentially expressed in our microarray-based screening, confirmed that two microRNAs, miR139-3p and miR-720, were upregulated in BD with respect to HC ($p \leq 0.05$, FDR <5%).

ROC analysis was performed to evaluate the predictive power of miR139-3p and miR-720 to discriminate BD from HC: the calculated AUC was 0.84 (95% CI, 0.71–0.98) for miR-139-3p and 0.87 (95% CI, 0.78–0.97) for miR-720. If miR-139-3p was combined with miR-720 the performance of the test increased [AUC of 0.92 (95% CI, 0.83–1)]. There were no differences in the PPV when miR-139-3p and miR-720 were used alone or in combination (PPV 0.97 for all the three tests), while on the contrary combination of miR-139-3p and miR-720 displayed a better NPV (NPV of 0.60 for miR-139-3p plus miR-720) with respect to each single test (NPV of 0.46 and 0.47, for miR-139-3p and miR-720, respectively) (Fig. 1).

The sensitivity, specificity, AUC (95% CI), positive predictive value (PPV) and negative predictive value (NPV) of miR-139-3p and miR-720 alone and in combination in the validation cohort are summarized in Fig. 1 along with counts of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) tests.

Of great importance, a post-hoc analysis performed stratifying the whole BD group on the basis of the presence of active clinical features, showed that 9 out of 13 microRNAs deregulated in the microarray screening (miR-127, miR-370, miR-139-3p, miR-720, miR-330, miR-200b, miR-199a-3p, miR-224 and

miR-98) were differentially expressed in PBMCs from active BD with respect to their counterpart from quiescent BD (Table III; Fig. 2). We supposed that inflammatory state would greatly account for this difference driving microRNAs upregulation in active BD patients.
On the contrary, post-hoc analysis did not show significant difference in microRNAs expression according to BD clinical subset, HLA-B51/HLA-B*51:01 positivity and current therapy (data not shown). Expression profiles of microRNAs of the *a priori* selected list is presented as supplementary material [see Additional file 2].
Table IV and Figure 3 report genes associated with susceptibility to BD and the respective microRNAs validated and predicted interactions.

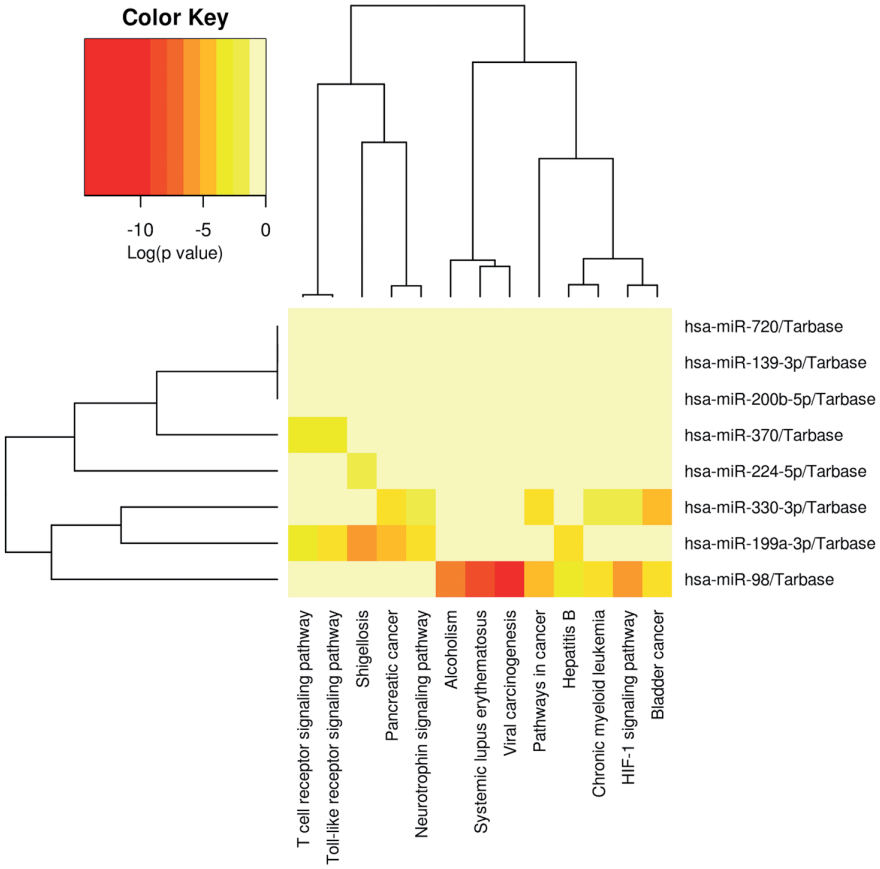


Fig. 3. Deregulated microRNAs *vs*. pathways heatmap.
Clustering is based on significance levels. Darker colours represent lower significance values, DIANA-miRPath v 2.0 sever; pathway union.

Additional file 1. List of *"a priori"* selected microRNAs with their putative targets relevant for BD pathogenesis.

microRNA	*Putative targets*
Let-7a, Let-7b, Let-7e, Let-7f, Let-7g	IL-10, TLR-pathway
hsa-miR23b	IL-17, NOTCH-1, Th17
hsa-miR223	Granulocyte function
hsa-miR155	TLR-pathway
hsa-miR181a	Inflammatory response, autoimmune diseases
hsa-miR146a	IL-10, TLR-pathway

Discussion

microRNAs are emerging as fundamental regulators of inflammation and innate and adaptive immune response molecular pathways and have been shown to be associated with several rheumatic diseases (11). However, data about epigenetic regulation in BD are limited (12-15) and no information coming from comprehensive microRNA profiling in BD are available. To the best of our knowledge, this is the first study reporting a large array-based analysis of microRNAs expression in BD.
From an epidemiological point of view, data about microRNAs expression in BD in our island could be of particular interest. The Sardinian population is characterised by a homogeneous genetic background with little gene inflow and a peculiar high frequency of autoimmune diseases (*e.g.* type 1 diabetes and multiple sclerosis) (24, 25). Lower background genetic variation resulting in reduced background variation in microRNAs expression could contribute to maximise the chances of identifying *true* positive associations. Furthermore, from a pathophysiological perspective, it is likely that Sardinian BD patients share more homogeneous genetic traits (25) whose identification may suggests a defined pathogenetic mechanism, but with a less universal significance than that detectable in non-isolated populations, since it is determined by the interaction of a lower number of gene variants with a reduced number of environmental variables.
The main result of our study is the demonstration that two microRNAs, miR-139-3p and miR-720, are significantly upregulated in PBMCs of BD patients with respect to their counterpart from HC. Noteworthy, the expression of these two microRNAs did not seem to be dependent on disease activity suggesting that miR-139-3p and miR-720 may related to BD susceptibility.
Although ROC curve analysis displayed that level of miR-139-3p and miR-720 in PBMCs could discriminate BD patients from HC with elevated accuracy, and that combination of these two miR may have even higher performance, further evaluation are needed to confirm these very preliminary results.
In silico exploration of putative targets of miR-139-3p and miR-720 did not revealed a large number of regulatory interactions targeting genes associated to BD predisposition. We identified RELT (receptor expressed in lymphoid tissues) as an interesting candidate target gene of miR-139-3p: RELT encodes a member of the TNF receptor superfam-

Additional file 2. Significantly deregulated "a priori selected microRNAs" in PBMCs from active BD vs quiescent BD.

miR Unique ID	Chromosome location	FDR	Style	Fold change	*p* value
hsa-miR-23b-000400	chr9: 97847490-97847586 [+]	0.00128	Up	2.59	0.0002141
hsa-miR-223-000526	chrX: 65238712-65238821 [+]	0.00155	Up	2.82	0.0003452
hsa-let-7a-000377	chr9: 96938239-96938318 [+] chr11: 122017230-122017301 [-]	0.00158	Up	2.12	0.0004379
hsa-miR-146a-000468	chr5: 159912359-159912457 [+]	0.00181	Up	2.31	0.0006035
hsa-let-7b-000378	chr22: 46509566-46509648 [+]	0.00552	Up	2.19	0.0010968
hsa-let-7f-00038	chr9: 96938629-96938715 [+] chrX: 53584153-53584235 [-]	0.00613	Up	2.09	0.0040854
hsa-miR-155-002623	chr21: 26946292-26946356 [+]	0.0165	Up	1.49	0.0137845

ily which is expressed in cells of the hematopoietic lineage and may play a role in T cells differentiation and activity (26). This observation could be of particular interest, considering that several lines of evidence points toward a role of T cells, especially Th17, in BD onset and maintenance (15, 27-30). This could be worthy of mention in the light of evidence that downregulated miR-23b expression in $CD4^+$T cells from active BD patients relieves negative control of Notch-1 (a type 1 transmembrane protein family member) signalling leading to expansion of Th17 cells (15).

An interesting secondary finding of our study was that significantly different microRNAs expression patterns exist between active and quiescent BD patients suggesting microRNAs as regulators of molecules/molecular pathways involved in inflammatory and immune process in BD.

Recurrent innate immune system activation triggered by environmental antigens on a genetic favourable background has been claimed to be one of the main pathogenic mechanisms in BD (31). In agreement with this model, TLR have been reported to be iperexpressed in blood and tissue samples from BD patients (32-34) and polymorphisms in TLR encoding genes have been associated to susceptibility to disease (9, 10). Noteworthy, among the microRNAs upregulated in PBMCs from our active BD patients we observed that miR-23, miR-200b, miR-98 and miR-224 are predicted to target TLR-2 gene.

Zhou *et al*. (14) found a significant lower expression of miR-155 in PBMCs of BD patients with active uveitis while we found a significant higher expression of miR-155 in PBMCs of active BD patients irrespective from clinical subtype. It is therefore reasonable that the different patient selection strategy may account for this opposite result.

According with our results in BD patients, upregulation of miR-155 has been reported in other rheumatic diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and type I diabetes mellitus suggesting its possible role as a mediator of immune and inflammatory response (35).

IL-10 is a Th2-lineage cytokine with a potent anti-inflammatory activity. Recent GWAS have identified the independent association of IL-10 with BD risk (5, 6). miR-98 has been reported to negatively regulate IL-10 production after exposure to lipopolysaccharide (36) thus dampering inflammatory response. Upregulation of miR-98 in PBMCs from our patients with active BD may suggest that this negative feedback control of inflammation during active phase of BD does not efficiently work giving to persistence of high grade inflammation.

Among candidate susceptibility genes is the gene encoding for ERAP1 (endoplasmic reticulum aminopeptidase 1) that show epistatic interactions with HLA-B51 gene (10). ERAP1 may be involved in BD pathogenesis by cutting proteins into small peptides fitting with HLA-B51 molecule (10). Of interest, gene sequence of ERAP1 is a predicted target of miR-330 from our list of miR upregulated in active BD.

Beyond these speculative hypotheses, the evidence of several upregulated microRNAs in PBMC from patients with active versus quiescent BD is difficult to interpret mainly because microRNAs, frequently but not always, downregulate the expression of their target genes. It is possible to speculate that the activation of the inflammatory process induces the expression of genes involved in inflammation together with a discrete subset of microRNA working as both positive and negative regulators of inflammatory process.

Some limitations of this study should be discussed. First, this was designed as a preliminary investigational study, thus the expression of candidate target genes has not been tested.

Second, larger number of samples in the discovery phase would have probably allowed the definition of a better panel of microRNAs for the subsequent phase of qRT-PCR validation. In particular, the inclusion of control populations affected by other disease and an higher number of active BD cases would have enabled candidates microRNAs to be validated separately according to other pathogenetic mechanisms and to the status of BD disease activity, respectively. It should be remarked, indeed, that the different proportion of active BD patients, even if not statistically significant, may have affected microRNAs expression in the discovery and validation cohorts.

Third, all BD patients were under immunosuppressive therapy at the time of sampling, thus we cannot rule out an effect of these drugs on microRNAs differential expression.

Finally, the universal significance of the results of this investigation needs to be tested by further studies in different populations. In fact Sardinian pop-

ulation is a genetic isolate with some highly conserved genetic traits which are the byproducts of an ancestral founder effect, genetic drift, endogamy and millenary environmental selective pressure, namely by malaria endemic, or of lethal epidemics from other microbial agents.

References

1. SAKANE T, TAKENO M, SUZUKI N, INABA G: Behçet's disease. *N Engl J Med* 1999; 341: 1284-91.
2. PIGA M, MATHIEU A: The Behçet's disease geoepidemiology: possible role of a dual microbial-driven genetic selection. *Clin Exp Rheumatol* 2014; 32 (Suppl. 84): S123-9.
3. FIETTA P: Behçet's disease: familial clustering and immunogenetics. *Clin Exp Rheumatol* 2005; 23 (Suppl. 38): S96-105.
4. PIGA M, MATHIEU A: Genetic susceptibility to Behçet's disease: role of genes belonging to the MHC region. *Rheumatology* (Oxford) 2011; 50: 299-310.
5. REMMERS EF, COSAN F, KIRINO Y *et al*.: Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behçet's disease. *Nature Genetics* 2010; 42: 698-702.
6. MIZUKI N, MEGURO A, OTA M *et al*.: Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behçet's disease susceptibility loci. *Nature Genetics* 2010; 42: 703-6.
7. HOU S, YANG Z, DU L *et al*.: Identification of a susceptibility locus in STAT4 for Behçet's disease in Han Chinese in a genome-wide association study. *Arthritis Rheum* 2012; 64: 4104-13.
8. HOU S, XIAO X, LI F *et al*.: Two-stage association study in Chinese Han identifies two independent associations in CCR1/CCR3 locus as candidate for Behçet's disease susceptibility. *Hum Genet* 2012; 131: 1841-50.
9. HORIE Y, MEGURO A, OTA M *et al*.: Association of TLR4 polymorphisms with Behçet's disease in a Korean population. *Rheumatology* (Oxford) 2009; 48: 638-42.
10. KIRINO Y, BERTSIAS G, ISHIGATSUBO Y *et al*.: Genome-wide association analysis identifies new susceptibility loci for Behçet's disease and epistasis between HLA-B*51 and ERAP1. *Nat Genet* 2013; 45: 202-7.
11. CERIBELLI A, YAO B, DOMINGUEZ-GUTIERREZ PR *et al*.: MicroRNAs in systemic rheumatic diseases. *Arthritis Res Ther* 2011; 13: 229.
12. QI J, HOU S, ZHANG Q *et al*.: A functional variant of pre-miRNA-196a2 confers risk for Behçet's disease but not for Vogt-Koyanagi-Harada syndrome or AAU in ankylosing spondylitis. *Hum Genet* 2013; 132: 1395-404.
13. ZHOU Q, HOU S, LIANG L *et al*.: MicroRNA-146a and Ets-1 gene polymorphisms in ocular Behçet's disease and Vogt-Koyanagi-Harada syndrome. *Ann Rheum Dis* 2014; 73: 170-6.
14. ZHOU Q, XIAO X, WANG C *et al*.: Decreased microRNA-155 expression in ocular Behçet's disease but not in Vogt Koyanagi Harada syndrome. *Invest Ophthalmol Vis Sci* 2012; 53: 5665-74.
15. QI J, YANG Y, HOU S *et al*.: Increased Notch pathway activation in Behçet's disease. *Rheumatology* (Oxford) 2014; 53: 810-20.
16. CRITERIA FOR DIAGNOSIS OF BEHÇET'S DISEASE: International Study Group for Behçet's Disease. *Lancet* 1990; 335: 1078-80.
17. TarBase ver. 6.0 [http://www.microrna.gr/tarbase]
18. VERGOULIS T, VLACHOS IS, ALEXIOU P *et al*.: Tarbase 6.0: Capturing the Exponential Growth of miRNA Targets with Experimental Support. *Nucl Acids Res* 2012; 40: D222-D229.
19. DIANA microT-CDS [http://www.microrna.gr/microT-CDS]
20. PARASKEVOPOULOU MD, GEORGAKILAS G, KOSTOULAS N *et al*.: DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic Acids Res* 2013; 41: W169-73.
21. miRecords [http://mirecords.umn.edu/miRecords]
22. miRPath v2.0 [ttp://www.microrna.gr/miRPathv2]
23. VLACHOS IS, KOSTOULAS N, VERGOULIS T *et al*.: DIANA miRpath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res* 2012; 40: W498-504.
24. CONTU D, MORELLI L, SANTONI F *et al*.: Y chromosome based evidence for pre-neolithic origin of the genetically homogeneous but diverse Sardinian population: inference for association scans. *PLoS One* 2008; 3: e1430.
25. PIGA M, PALADINI F, LAI S *et al*.: Genetics of Behçet's disease in Sardinia: two distinct extended haplotypes habour the B*51 allele in the normal population and in patients. *Clin Exp Rheumatol* 2012; 30 (Suppl. 72): S51-S56.
26. SICA GL, ZHU G, TAMADA K *et al*.: RELT, a new member of the tumor necrosis factor receptor superfamily, is selectively expressed in hematopoietic tissues and activates transcription factor NF-kappaB. *Blood* 2001; 97: 2702-7.
27. NA SY, PARK MJ, PARK S, LEE ES: Up-regulation of Th17 and related cytokines in Behçet's disease corresponding to disease activity. *Clin Exp Rheumatol* 2013; 31 (Suppl. 77): 32-40.
28. GÜNDÜZ E, TEKE HU, BILGE NS *et al*.: Regulatory T cells in Behçet's disease: is there a correlation with disease activity? Does regulatory T cell type matter? *Rheumatol Int* 2013; 33: 3049-54.
29. PARLAKGUL G, GUNEY E, ERER B *et al*.: Expression of regulatory receptors on γδ T Cells and their cytokine production in Behçet's disease. *Arthritis Res Ther* 2013; 15: R15.
30. HAMZAOUI K: Th17 cells in Behçet's disease: a new immunoregulatory axis. *Clin Exp Rheumatol* 2011; 29 (Suppl. 67): S71-6.
31. ZIERHUT M, MIZUKI N, OHNO S *et al*.: Immunology and functional genomics of Behçet's disease. *Cell Mol Life Sci* 2003; 60: 1903-22.
32. LIU X, WANG C, YE Z, KIJLSTRA A, YANG P: Higher Expression of Toll-like Receptors 2, 3, 4, and 8 in Ocular Behçet's Disease. *Invest Ophthalmol Vis Sci* 2013; 54: 6012-7.
33. DO JE, KWON SY, PARK S, LEE ES: Effects of vitamin D on expression of Toll-like receptors of monocytes from patients with Behçet's disease. *Rheumatology* (Oxford) 2008; 47: 840-8
34. SEOUDI N, BERGMEIER LA, HAGI-PAVLI E *et al*.: The role of TLR2 and 4 in Behçet's disease pathogenesis. *Innate Immun*, in press
35. PICASCIA A, GRIMALDI V, PIGNALOSA O *et al*.: Epigenetic control of autoimmune diseases: From bench to bedside. *Clin Immunol* 2015; 157: 1-15.
36. LIU Y, CHEN Q, SONG Y *et al*.: MicroRNA-98 negatively regulates IL-10 production and endotoxin tolerance in macrophages after LPS stimulation. *FEBS Lett* 2011; 585: 1963-8.