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Original Article

Circulating microRNA-125b expression predicts clinical response in patients with rheumatoid arthritis treated by tumor necrosis factor inhibitors

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Abstract: This study was aimed to investigate the correlation of serum microRNA-125b (miR-125b) expression and clinical response to tumor necrosis factor inhibitors (TNFi) in RA patients. 89 moderate to severe RA patients receiving etanercept (ETN) of infliximab (IFX) treatment were recruited in this prospective study. 40 health volunteers with matched age, gender were also included as health controls (HC). Serum samples were collected from all participants at baseline and miR-125b expression was measured by RT-PCR assay. A decrease of 1.2 points in DAS28 at week 30 compared with baseline was defined as clinical response. Serum miR-125b expression was decrease in RA patients compared with HC, P<0.001, with a diagnostic value AUC 0.722, 95% CI: 0.626-0.819 by Receiver Operating Characteristic (ROC) curve. Serum miR-125b level at baseline was increased in responders compared to non-responders, P=0.001. The ROC curve analysis illuminated miR-125b had a prognostic value in predicting clinical response in RA patients treated by TNFi, with AUC: 0.725, 95% CI: 0.605-0.849. After subgroup analysis, we found serum miR-125b was higher in responders than in non-responders of ETN treated subgroup, with AUC: 0.775, 95% CI: 0.622-0.927 by ROC curve, while no difference was found in IFX treated subgroup. In addition, univariate and multivariate logistic analysis exhibited that serum miR-125b high expression was an independent predictive factor for clinical response in ETN treated RA patients, with OR 5.332 95% CI 1.315-21.625, P=0.019. Circulating miR-125b expression could be served as a feasible and promising diagnostic and prognostic biomarker in RA patients treated by TNFi.

Keywords: Serum, miR-125b, rheumatoid arthritis, predict, clinical response

Introduction

Rheumatoid arthritis (RA) is a chronically inflammatory autoimmune disease which affecting approximately 1% of the general population with unknown etiology [1]. Due to much progress made in early diagnosis, treatment strategy, imaging and new drugs, the prognosis of RA has improved greatly in recent decades, especially benefits from the development of tumor necrosis factor inhibitors (TNFi) which substantially improves patient's clinical and radiographic outcome in RA [2, 3]. However, almost 20-30% of patients don't respond to TNFi treatment [4, 5]. Therefore, it's of great need to explore promising biomarkers for clinical response in RA patients treated by TNFi.

One of the factors influence the lack of treatment response might be the epigenetic regulation of gene expression, which is associated with non-coding RNAs, most importantly, microRNAs (miRNA) [6]. MiRNAs, composed of 21-25 nucleotides, play a crucial role in pathogenesis, progress and prognosis of RA, mainly through the regulation by targeting mRNAs for translational repression or degradation [6].

MiRNA-125b (MiR-125b), a member of miR-125 family, is reported to be a regulator of inflammatory cytokines such as TNF-α and interferon (IFN)-γ [7, 8]. Veronika Hruskova et al illuminates that the expression of miR-125b in the peripheral blood mononuclear cells (PBMC) and plasma is associated with early RA risk and disease activity [9]. Isabelle Duroux-Richard et al presents that serum miR-125b high expression predicts clinical response in established RA patients treated by rituximab [10]. However, the role of circulating miR-125b expression in prog-

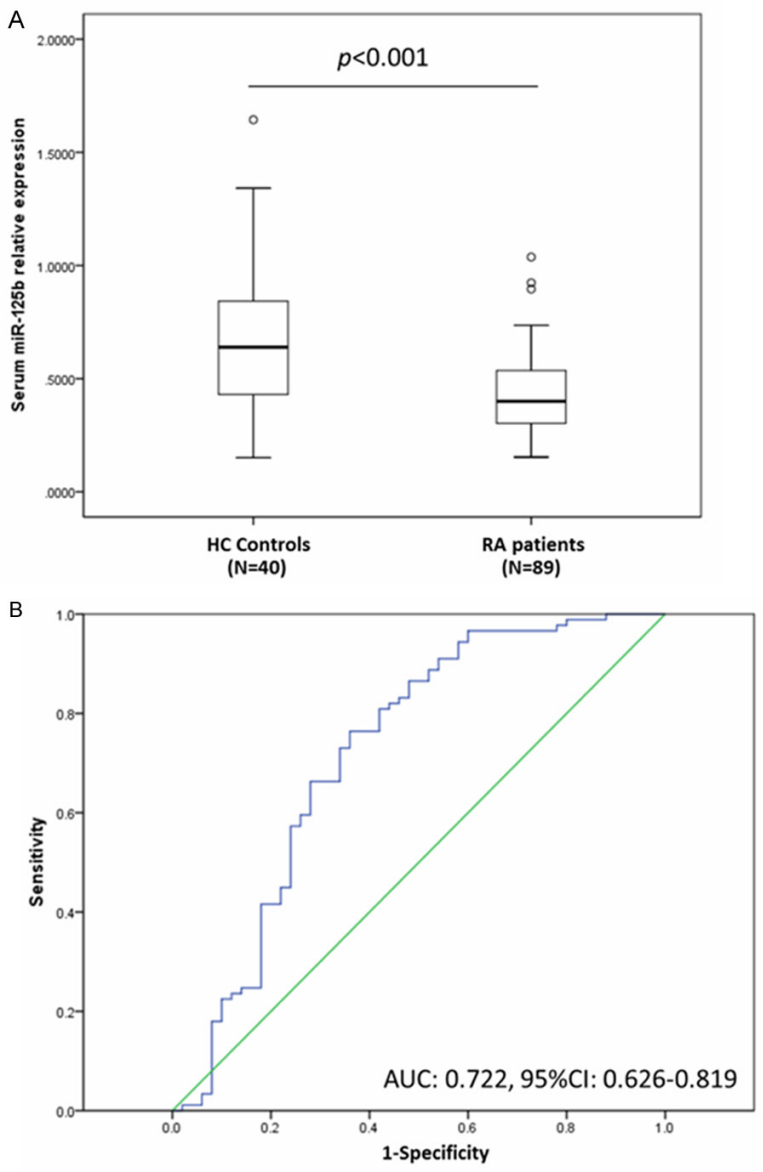

Figure 1. A. Serum miR-125b expression in RA patients and HC controls; B. ROC curve of serum miR-125b level for diagnosis of RA.

nosis of RA patients treated by TNFi is still obscure. So this study was aim to investigate the correlation of serum miR-125b expression and clinical response to TNFi in RA patients.

Materials and methods

Participants

89 moderate to severe RA patients at Department of Rheumatology and Immunology in Yueyang Hospital Integrated Traditional Chinese and Western Medicine Affiliated to Shanghai University of TCM, between 2011/5/31 and 2015/12/31 were recruited in this prospective study. Inclusive criteria: age >18; diagnosed with RA according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria [11]; moderate to severe disease activity (defined as 28-joints Disease Activity score (DAS28) ≥3.2). Exclusive criteria: Treatment with etanercept (ETN), infliximab (IFX), other TNF-α inhibitors or other biologic agents within 3 months; severe ankylosis of the joints; pregnant or lactating women. All the RA patients received ETN or IFX treatment for 30 weeks. 40 health volunteers at Physical Examination Department with matched age, gender were also included in this study as health controls (HC).

This study was approved by the Ethics Committee of Yueyang Hospital Integrated Traditional Chinese and Western Medicine Affiliated to Shanghai University of TCM. Both patients and volunteers provided written informed consent.

Clinical response

DAS28 was calculated before and after treatment. A decrease of 1.2 points in DAS28 at week 30 compared with baseline was defined as clinical response according to the EULAR response criteria [12].

RT-PCR

Serum samples were collected from all participants at baseline (RA patients before treatment and HC patients undergoing physical examination), and total RNA was isolated using Trizol Reagent (Invitrogen, USA). RNA was reversely transcribed to complementary DNA by iScript cDNA synthesis kit (Bio-Rad, USA). RT-PCR assay was performed using iQ™ SYBR® Green Supermix kit (Bio-Rad, USA). All the processes were performed according to manufac-

Table 1. Characteristics of analyzed RA patients treated by TNFi

Parameters	Final analysed patients (n=81)
Age (years)	48±11
Gender	
Male (%)	24 (30%)
Female (%)	57 (70%)
Disease Duration (months)	47 (39-75)
Rheumatoid Factor (RF)	
Positive (%)	63 (78%)
Negative (%)	18 (22%)
Anti-cyclic citrullinated peptide (CCP)	
Positive (%)	54 (67%)
Negative (%)	27 (33%)
ESR (mm/h)	35.2 (28.7-43.1)
CRP (mg/l)	29.1 (22.5-38.3)
DAS28	5.8±1.4
TNF inhibitor treatment	
ETN	52 (64%)
IFX	29 (36%)

Data was presented as Mean values ± SD, median (25^{th}-75^{th}) or counts (percentage). ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; ETN, etanercept; IFX, infliximab.

turer's instructions and standard experimental criteria. Relative expression of miR-125b was calculated referring to the threshold cycle (CT), and was normalized to U6 as internal reference. Data were analyzed by $2^{-\Delta\Delta Ct}$ method.

Statistics

Statistical analysis was performed using the SPSS 22.0 program. Data between two groups were compared by the Wilcoxon rank sum test or Chi-square test. Receiver Operating Characteristic (ROC) curve was performed to evaluate the diagnostic and prognostic value of miR-125b in RA. Univariate logistic regression model was used to analyze the factors which influence clinical response, and all factors with a p value ≤0.1 were further analyzed by multivariate logistic regression model. A p value <0.05 was considered significant and P<0.01 as extremely significant.

Results

Decreased serum miR-125b expression in RA patients

Serum miR-125b expression level was decrease in RA patients (0.401 (0.230-0.539)) compared with age and sex matched health controls (0.639 (0.418-0.852)), P<0.001 as presented in **Figure 1A**, which indicated its inflammation suppression role in RA. ROC curve presented that miR-125b level could distinguish RA from health control with an area under curve (AUC) 0.722, 95% CI: 0.626-0.819 (**Figure 1B**).

Elevated serum miR-125b level at baseline in responders than non-responders

11 of 89 (12.4%) RA patients didn't complete a 30 week TNFi therapy, among which 4 cases due to lost follow-up, 2 cases withdrawing the informed consent, 2 cases infection, 3 cases unsatisfied efficacy. The 3 cases of unsatisfied efficacy were included into the final analysis for clinical response as non-responders while other 8 withdrawing cases excluded from the analysis.

Baseline characteristics of 81 analyzed RA patients were presented in **Table 1** with age 48±11 years, female 57 cases (70%) and disease duration 47 (39-75) months. 52 patients were treated by ETN while 29 patients by IFX.

Among 81 RA patients, 55 cases (68%) that achieved a decrease of DAS 28 at week 30 from baseline were included into responders, while other 26 cases (32%) included into non-responders.

Serum miR-125b level at baseline was increased in responders (0.439 (0.350-0.578)) compared to non-responders (0.310 (0.222-0.409)), P=0.001 as presented in **Figure 2A**. The ROC curve analysis illuminated miR-125b had a prognostic value in predicting clinical response in RA patients treated by TNFi, with an AUC: 0.725, 95% CI: 0.605-0.849, shown in **Figure 2B**. While the median of miR-125b (0.402) in analyzed patients was selected as cut off value, the predictive sensitivity was 62.4% and specificity was 76.9%.

Serum miR-125b level at baseline predicted clinical response in ETN treated patients but not IFX treated patients

We further divided the patients into ETN treated (52 cases) and IFX treated subgroups (29 cases) to investigate the predictive value of baseline serum miR-125b expression in specific TNFi drug. As presented in **Figure 3A**, in ETN treated subgroup, serum miR-125b was higher (0.449 (0.367-0.576)) in responders

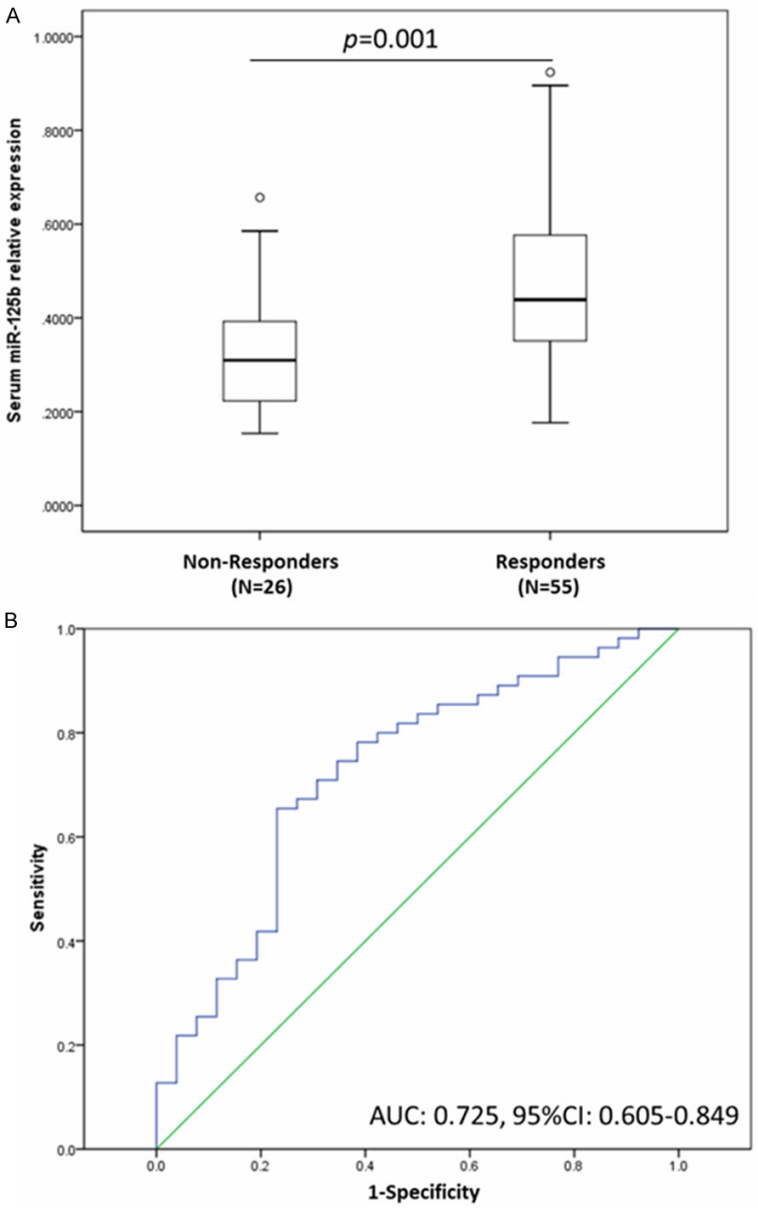

Figure 2. A. Baseline serum miR-125b expression in responders and non-responders of TNFi treated RA patients; B. ROC curve of baseline serum miR-125b level for predicting clinical response in TNFi treated RA patients.

than in non-responders (0.254 (0.208-0.399)), with AUC: 0.775, 95% CI: 0.622-0.927 by ROC curve analysis (**Figure 3B**). While the median of miR-125b (0.402) in analyzed patients was selected as cut off value, the predictive sensitivity was 61.8% and specificity was 77.8%. However, no difference was found between responders and non-responders in patients treated by IFX, as shown in **Figure 3C**, **3D**. These results indicated the prognostic role of miR-125b in ETN treated RA patients but not IFX treated ones.

Analysis of factors at baseline predicting the clinical response in RA patients treated by ETN

Baseline serum level of miR-125b was divided into two groups with cut off value as the median of miR-125b (0.402) in total analyzed patients: miR-125b high expression >0.402 and miR-125b low expression ≤0.402. Age, disease duration, ESR, CRP and DAS 28 at baseline were as well divided into two groups with cut off value set as the median value of their own in total analyzed patients.

Univariate logistic analysis exhibited that serum miR-125b high expression was associated with a higher possibility to achieve clinical response compared with low expression in ETN treated RA patients, with odds ratio (OR) 5.654, 95% CI 1.527-20.931, P=0.009, **Table 2**. In addition, Anti-cyclic citrullinated peptide (Anti-CCP) positive could also predict clinical response.

All factors with a *p* value ≤0.1 in univariate logistic model were further analyzed by multivariate logistic regression model, which indicated serum miR-125b high expression was an independent predictive factor for clinical response in RA patients treated by ETN, with OR 5.332 95% CI 1.315-21.625, P=0.019, **Table 2**.

Discussion

Our study presented that (1) Serum miR-125b expression was decreased in RA patients compared with HC. (2) Serum miR-125b expression as baseline was elevated in responders than in non-responders to TNFi treatment in RA patients with a good predictive value by ROC curve. (3) Further analysis indicated serum miR-

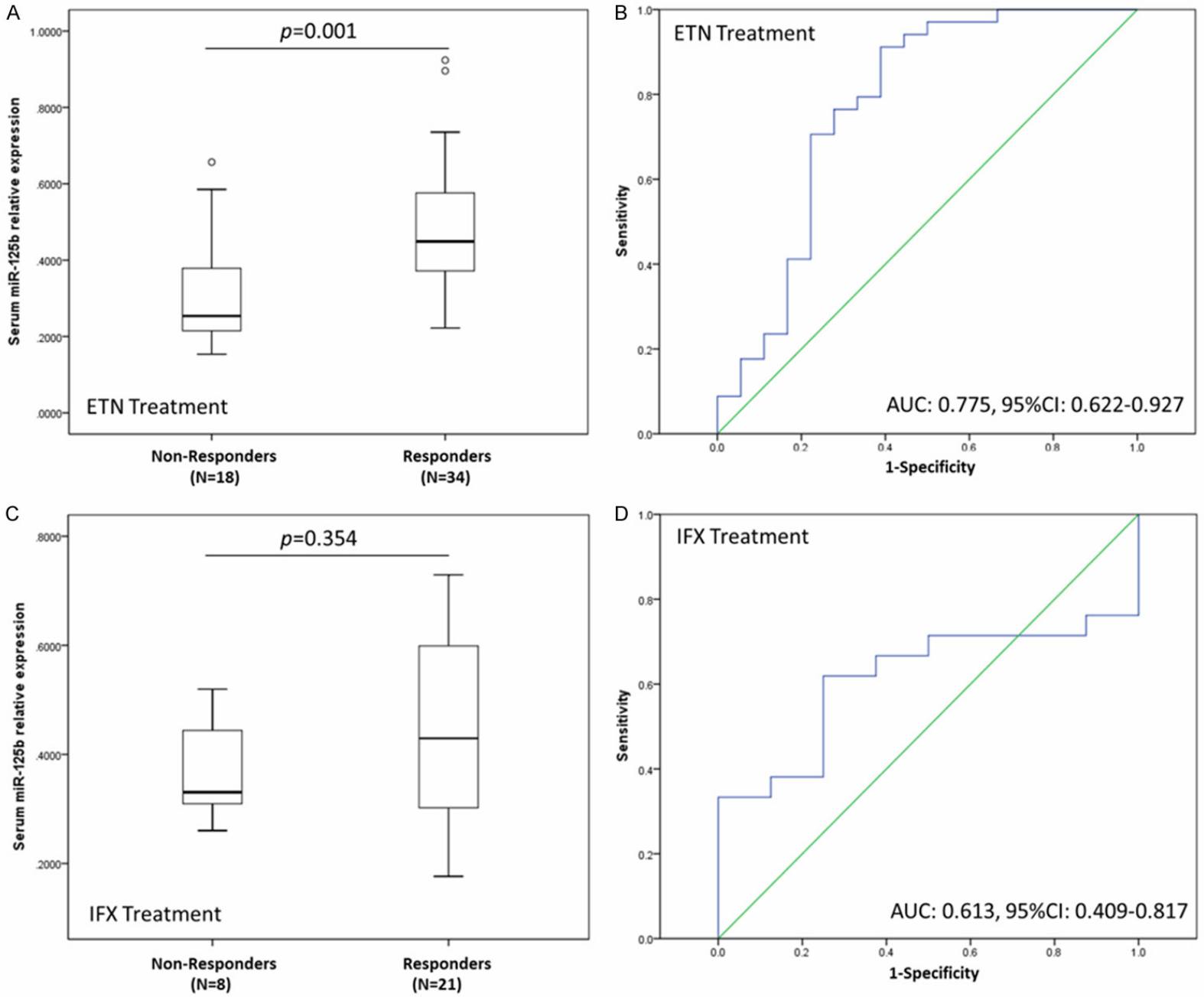
A
p=0.001
Serum miR-125b relative expression
1.0000
.8000
.6000
.4000
.2000
.0000
ETN Treatment
Non-Responders
(N=18)
Responders
(N=34)
B
ETN Treatment
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
AUC: 0.775, 95%CI: 0.622-0.927
0.0
0.2
0.4
0.6
0.8
1.0
1-Specificity
C
p=0.354
Serum miR-125b relative expression
.8000
.6000
.4000
.2000
IFX Treatment
Non-Responders
(N=8)
Responders
(N=21)
D
IFX Treatment
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
AUC: 0.613, 95%CI: 0.409-0.817
0.0
0.2
0.4
0.6
0.8
1.0
1-Specificity

Figure 3. A. Baseline serum miR-125b expression in responders and non-responders treated by ETN; B. ROC curve of baseline serum miR-125b level for predicting clinical response in ETN treated RA patients; C. Baseline serum miR-125b expression in responders and non-responders treated by IFX; D. ROC curve of baseline serum miR-125b level for predicting clinical response in IFX treated RA patients.

Table 2. Analysis of Factors at baseline predicting the clinical response in ETN treated RA patients

Parameters	Univariate Logistic model				Multivariate Logistic model			
	p value	OR	95% CI		*p* value	OR	95% CI	
			Lower	Higher			Lower	Higher
MiR-125b (High vs Low)	0.009	5.654	1.527	20.931	0.019	5.332	1.315	21.625
Age (≤46 vs >46 years)	0.247	1.990	0.621	6.379	-	-	-	-
Gender (Female vs Male)	0.561	0.711	0.226	2.241	-	-	-	-
Disease Duration (≤47 vs >47 months)	0.325	1.786	0.563	5.660	-	-	-	-
RF (Positive vs Negative)	0.235	2.019	0.634	6.433	-	-	-	-
Anti-CCP (Positive vs Negative)	0.038	3.714	1.078	12.797	0.194	2.463	0.631	9.607
ESR (High vs Low)	0.253	2.000	0.609	6.564	-	-	-	-
CRP (High vs Low)	0.088	2.925	0.853	10.025	0.118	3.002	0.758	11.889
DAS 28 (High vs Low)	0.175	2.245	0.698	7.219	-	-	-	-

Data was presented as *p* value, odds ratio (OR) and 95% CI. RF, Rheumatoid Factor; Anti-CCP, Anti-cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints. Univariate Logistic model was used to analyze the factors at baseline in predicting clinical response in ETN treated RA patients. Furthermore, all factors with a P≤0.1 in univariate model were analyzed by multivariate Logistic model. A P<0.05 was considered significant and P<0.01 extremely significant.

125b level at baseline predicted clinical response only in ETN treated patients but not in IFX treated patients, and miR-125b high expression was an independent prognostic factor for clinical response in ETN treated RA patients.

miRNAs have been demonstrated to be associated with the pathogenesis of RA in recent years [6]. MiR-16 is reported to be up-regulated in PBMC, plasma of RA patients and is capable to target 3'-UTR of TNF-α [13, 14]. MiR-21 is decreased in PBMC and $CD4^+$ T cells of RA patients, and induces an increase of T-helper 17 (Th17) cells, forkhead box P3 (FOXP 3) mRNA levels as well as up regulating signal transducer and activator of transcription 3 (STAT3) expression [15]. A decrease of miR-23b expression is revealed in RA patients compared with osteoarthritis (OA) patients and HC, and miR-23b suppresses L-17-associated autoimmune inflammation by targeting TGF-β-activated kinase 1/MAP3K7 binding protein 2 (TAB2), TAB3 and inhibitor of nuclear factor κ-B kinase subunit α (IKK-α) [16].

miR-125b, a member of miR-125 family, could activates the nuclear factor-κB (NF-κB) pathway by targeting tumor necrosis factor alpha-induced protein 3 (TNFAIP3) synergized with miR-125a, which indicates its proinflammatory role. On the other side, miR-125b has been disclosed as a suppressor of inflammatory cytokines such as TNF-α, IFN-γ and Chemokine CCL4 [7, 8, 17], which suggests its inflammation inhibition role. These implied the dual role of miR-125a in inflammation. In the present study, we observed serum miR-125b expression was decreased in RA patients compared to HC, which in accordance of the previous results in PBMC and plasma sample of RA patients by Veronika Hruskova et al [9]. But Isabelle Duroux-Richard et al showed a contrary result: miR-125b was elevated in total blood and serum of RA patients compared with OA patients and HC [10]. This discrepancy may be explained by the dual role of miR-125b in inflammation and disease duration differences: Isabelle Duroux-Richard included patients were mainly established RA patients with mean disease duration 16 years, while out study recruited both early and established RA patients with median disease duration 4 years.

PBMC miR-125b high expression was reported to be a novel biomarker for treatment response at 3 months in early RA patients [9]. While Isabelle Duroux-Richard et al presents that serum miR-125b level predicts clinical response to rituximab treatment at 3 months

established RA patients [10]. In line with the above study, we found serum miR-125b high expression at baseline could predict clinical response in TNFi treated RA patients at 30 weeks with a high AUC.

Furthermore, we divided the patients into ETN treated and IFX treated groups to lucubrate the predictive value of miR-125b on TNFi treatment in RA patients. We found that miR-125b expression at baseline could only predict clinical response in ETN treated group but not in IFX treated group. And univariate and multivatiate logistic analysis ascertained the independent role of serum miR-125b level in predicting clinical response of ETN treatment in RA patients. These results may be on account of: (1) Small size sample in IFX treated group. 29 patients were treated by IFX, and only 8 cases were non-responders. (2) Difference between receptor fusion protein and monoclonal antibody. ETN is a recombinant tumor necrosis factor receptor: Fc fusion protein while IFX is a chimeric anti-tumor necrosis factor alpha monoclonal antibody [18, 19].

In conclusion, this study indicated that circulating miR-125b expression could be served as a feasible and promising diagnostic and prognostic biomarker in RA patients treated by TNFi.

Disclosure of conflict of interest

None.

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