Higher Expression of Whole Blood MicroRNA-21 in Patients with Ankylosing Spondylitis Associated with Programmed Cell Death 4 mRNA Expression and Collagen Cross-linked C-telopeptide Concentration

Chun-Huang Huang, James Cheng-Chung Wei, Wei-Chiao Chang, Shang-Yan Chiou, Chia-Hsuan Chou, Yu-Jie Lin, Pei-Hsuan Hung, and Ruey-Hong Wong

ABSTRACT. ***Objective.*** Bone loss is a recognized feature of ankylosing spondylitis (AS). The binding of microRNA-21 (miR-21) to programmed cell death 4 (PDCD4) could inhibit the expression of PDCD4 and further induce the activation of osteoclasts. In the present study, we compared the difference in miR-21 expression between patients with AS and healthy controls, and evaluated the relationships of miR-21, PDCD4 mRNA, and bone erosion in patients with AS. The influences of nonsteroidal antiinflammatory drugs (NSAID) and disease-modifying antirheumatic drugs (DMARD) on the expressions of miR-21 and PDCD4 mRNA in patients with AS were also assessed.

Methods. Whole blood miR-21 and PDCD4 mRNA expression were evaluated by quantitative real-time PCR among 122 patients with AS and 122 healthy controls. The serum level of collagen cross-linked C-telopeptide (CTX) was measured using ELISA.

Results. When compared to controls, patients with AS had significantly higher levels of miR-21, PDCD4 mRNA, and CTX. MiR-21 expression was negatively correlated with PDCD4 mRNA expression in patients with AS who were taking neither NSAID nor DMARD. Interestingly, significantly positive correlations between miR-21 expression with PDCD4 mRNA expression ($r = 0.33$, $p = 0.01$) and CTX level ($r = 0.44$, $p < 0.01$) were observed in patients with AS who were taking sulfasalazine. Positive correlations of miR-21 and CTX level were also observed in AS patients with disease duration < 7.0 years ($r = 0.36$, $p = 0.004$) and active disease ($r = 0.42$, $p = 0.001$).

Conclusion. The expression of miR-21 might have a role in the development of AS. (First Release May 1 2014; J Rheumatol 2014;41:1104–11; doi:10.3899/jrheum.130515)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS MICRORNA-21 PROGRAMMED CELL DEATH 4
COLLAGEN CROSS-LINKED C-TELOPEPTIDE

Ankylosing spondylitis (AS) is a chronic rheumatic disorder that usually starts at an early age and causes bone deformation in the long term[1]. It is characterized by inflammatory back pain and progresses slowly to cause motion restriction in the cervical, thoracic, and lumbar spine. The HLA-B27 gene is strongly associated with AS[2]. However, the definite pathogenesis of AS is still unknown. Importantly, there are 2 enhanced but opposite types of bone remodeling taking place in AS. One is pathologic new bone formation in the vertebrae, zygapophyseal joints, and ligamentous apparatus; another is excessive loss of trabecular bone mass in the vertebral body, leading to osteoporosis. Osteoporosis is caused by bone resorption through osteoclasts rather than bone formation through osteoblasts[3]. The bone loss may present early in the disease course of AS, although patients with a longer disease duration and inactive disease are less likely to develop osteopenia or osteoporosis[4].

MicroRNA (miRNA) are an abundant class of small RNA that play prominent roles in gene regulation[5]. Recently, miR-21 was identified as regulating various cells[6]. The binding of activator protein-1 (AP-1) transcription factor to the promoter of miR-21 has been shown to induce the expression of miR-21[7]. In particular, the expression of AP-1 in rheumatoid arthritis (RA) synovium was found to

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be constitutively upregulated to cause synovial hyperplasia and abnormal immune responses[8]. Further, receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis may be mediated by miR-21[9]. The expression of miR-21 was observed to be upregulated during RANKL-induced osteoclastogenesis in bone marrow-derived monocyte/macrophage precursors (BMM); whereas BMM deficient of DiGeorge syndrome critical region gene 8 or Dicer had significantly decreased miR-21 levels, and RANKL-induced osteoclastogenesis was impaired in those cells[9]. Therefore, increased expression of miR-21 might be associated with the activation of osteoclasts. In addition, programmed cell death 4 (PDCD4) has a suppressive function on AP-1 transactivation[10]. The evidence also shows PDCD4 may be one of the repressor proteins for osteoclastogenesis, and miR-21 expression is a critical prerequisite for downregulation of PDCD4, so the transcription factors for osteoclastogenesis are transcribed[9]. However, the expressions of miR-21 and PDCD4 mRNA have not been evaluated in patients with AS.

Previous studies observed that serum miR-21 expression in patients with hepatocellular carcinoma or chronic hepatitis[11] and breast cancer[12] were significantly greater than in healthy controls. These findings suggest elevated circulating miR-21 could come from specific tissue injury. However, there is overwhelmingly more cellular miRNA than extracellular miRNA[13], and serum or plasma miRNA might be sensitive to isolation procedures[14]. Therefore, whole blood is used to detect the expressions of miR-21 and PDCD4 mRNA in the current study. Bone resorption has been evaluated by assessing the serum collagen cross-linked C-telopeptide (CTX) in clinical practice[15]. Serum CTX was also used to apply the change of bone mineral density in patients with AS[16]. In our current study, the difference in whole blood miR-21 expression between patients with AS and healthy controls was measured, and the relationships between whole blood miR-21, PDCD4 mRNA expression, and serum CTX levels in patients with AS were evaluated. It has been reported that nonsteroidal antiinflammatory drugs (NSAID) might also modify the expression of PDCD4 in human colon carcinoma cells[17]. To our knowledge, there is no study that has evaluated the effects of NSAID and disease-modifying antirheumatic drugs (DMARD) on gene expression in patients with AS. In addition, the molecular mechanisms by which NSAID and DMARD exert their effects are still unclear. Therefore, evaluating the gene expressions of miR-21 and PDCD4 mRNA in patients with AS following NSAID and DMARD treatment could provide important information to understand some of the underlying mechanisms responsible for their pharmaceutical effect.

MATERIALS AND METHODS

Study subjects. The entire study conformed to the Declaration of Helsinki, and the design of the work and final report were performed with the approval of the institutional review board of Chung Shan Medical University Hospital. All study subjects were 16-65 years of age and signed an informed consent form. A total of 122 patients with AS were recruited from the rheumatology clinic at Chung Shan Medical University Hospital from October 2010 to March 2012. All patients were diagnosed using the modified New York criteria[18], and cognitive performance was not influenced by other diseases such as dementia. Sacroiliitis was confirmed by a qualified radiologist and AS diagnosis by a qualified rheumatologist. Detailed clinical histories were recorded, including age at initial symptom, family history of AS, medication history, and extraarticular manifestations. Age at the onset of AS symptom was defined as the time when the first symptom developed (axial symptom, peripheral arthritis, uveitis, or enthesitis). Delayed diagnosis was defined as the interval between the onset of the first symptom and the correct diagnosis of AS. Family history was defined as a first-degree relative with AS, inflammatory bowel disease (IBD), reactive arthritis, or psoriatic arthritis. The use of NSAID and DMARD was defined as drug administration for more than 3 months. In our present study, patients with AS were treated with different types of NSAID (aceclofenac, celecoxib, etoricoxib, and meloxicam) and DMARD [methotrexate and sulfasalazine (SSZ)]. Peripheral arthritis was defined as the presence of at least 1 swollen joint, and IBD was defined as the presence of the inflammatory condition of the colon and small intestine, including ulcerative colitis and Crohn disease. Uveitis was defined as the presence of inflammation of the middle layer of the eye involving unilateral, bilateral, or alternative patterns. These symptoms were ascertained by the rheumatologist, gastroenterologist, and ophthalmologist, respectively.

Cases were matched to controls in a 1:1 ratio for age (± 5 yrs) and sex. A total of 122 potential healthy controls was randomly selected from patients sequentially admitted to the same medical center for general physical examinations, and they had no rheumatic or autoimmune symptoms.

Bath Ankylosing Spondylitis indices. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Bath Ankylosing Spondylitis Global (BAS-G) were applied to evaluate the disease activity, physical function, and global wellbeing, respectively. The modified Chinese versions of BASDAI, BASFI, and BAS-G have good intraclass correlation and Cronbach's alpha coefficient[19].

Radiography. Lateral radiographs of the cervical, thoracic, and lumbar spine were acquired, and changes related to AS were assessed using the modified Stoke Ankylosing Spondylitis Spine Score (mSASSS)[20]. The score includes the anterior corners of vertebrae C2 to T1 and T12 to S1, which are graded with 0 to 3 points each (0 = normal, 1 = erosion, sclerosis or squaring, 2 = syndesmophyte, 3 = bridging syndesmophyte). The remaining thoracic spine is not included in the score. The scoring scale ranges from 0 to 72.

Biochemical assessments and HLA-B27. Peripheral blood was collected and centrifuged to separate serum and cells. Inflammatory indicators, including the erythrocyte sedimentation rate (ESR) and C-reactive protein, and leukocyte counts were assessed at the central laboratory of Chung Shan Medical University Hospital. The bone resorption marker CTX was measured using the serum CrossLaps 1-step ELISA kit (Osteometer Biotech) according to the manufacturer's instructions. Intraassay and interassay coefficients of variation were 3.0% and 10.6% for AS cases and 1.7% and 9.7% for controls, respectively. Carriage of HLA-B27 was determined by flow cytometry[21].

Reverse transcription (RT) and quantitative PCR (qPCR) of miR-21 and PDCD4 mRNA. Total RNA including small RNA was extracted from whole blood using a PAXgene Blood RNA kit (Qiagen) according to the manufacturer's protocol and treated with DNase (Qiagen). The absorbance at 260 and 280 nm of RNA was quantified spectrophotometrically with the NanoDrop ND-1000 (NanoDrop), and good integrity and purity of RNA were defined as having the A260/A280 ratio from 1.8 to 2.0. Samples were stored at –80°C. RT and qPCR kits made specifically for accurate miRNA

analysis (Applied Biosystems) were used to evaluate the expression of miR-21. The 15 μl RT reactions were performed using a TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) and incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then maintained at 4°C. For real-time PCR, 1 μl diluted RT products were mixed with 5 μl of 2 × Taqman PCR master mixture (No AmpErase UNG), 0.4 ml TaqMan MicroRNA Assay and 3.6 μl nuclease-free water in a final volume of 10 μl. All reactions were run on the Step One Plus Real Time PCR System (Applied Biosystems) using the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Real-time PCR was done in triplicate, including no-template controls. Relative expression of miR-21 was calculated using the comparative cycle threshold (CT; 2-DDCT) method[22] with RNU6B as the endogenous control to normalize the data.

The expression of PDCD4 mRNA was measured using a KAPA SYBR FAST One-Step quantitative real-time PCR Kit (Kapa Biosystems). Reactions were performed in 20 μl containing 1 μl mRNA, 10 μl 2 × KAPA SYBR FAST qPCR Master Mix, 0.4 μl forward primer, 0.4 μl reverse primer, 0.4 μl 10 mM dUTP, 0.4 μl 50 × KAPA RT mix, and 7.4 μl RNase-free H_2O. The protocol used was 5 min at 42°C and 3 min at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. GAPDH was used as an internal control. Target sequences were amplified with different sets of primers: PDCD4, forward 5′-AGT GGA TTA ACT GTG CCA ACC A-3′, reverse 5′-CTC CTG CAC CTT TCT TTG-3′; GAPDH, forward 5′-GGA GCC AAA AGG GTC ATC ATC-3′, reverse 5′-GAT GGC ATG GAC TGT GGT CAT-3′ The relative amounts of the target gene, standardized against the amount of GAPDH, were expressed as ΔCt = Ct(target) - Ct(GAPDH). The ratio of PDCD4 mRNA copies to GAPDH copies was then calculated as $2^{-\Delta\Delta CT}$[22].

Statistical analyses. The age at recruitment of patients with AS and healthy controls showed a normal distribution, and was thus presented by mean and SD, and compared by Student's t-test. Categorical variables were also presented by numbers (%), and the differences between patients with AS and healthy controls were compared by chi-square test. Kolmogorov-Smirnov normality test was used to evaluate the distribution of clinical features, leukocyte count, miR-21, PDCD4 mRNA, and CTX levels of patients with AS and healthy controls, and the distributions of these variables were skewed. These variables were further presented by median [first quartile (Q1) to third quartile (Q3)], and the differences between patients with AS and healthy controls were evaluated by Wilcoxon rank sum test. Taking a covariate (i.e., leukocyte count) into account, the robust regression was applied to compare the expression levels of miR-21, PDCD4 mRNA, and CTX between patients with AS and healthy controls. Further, the miR-21 expression, PDCD4 mRNA expression, CTX level, and mSASSS were compared by different treatment patterns in patients with AS using the Wilcoxon rank sum test. Spearman rank correlation was applied to test the correlations between miR-21 expression and the CTX level in patients with AS. After adjusting for the effects of confounding factors, we also calculated the partial correlations of miR-21 expression, PDCD4 mRNA expression, CTX level, and mSASSS with BAS indices and laboratory tests in patients with AS stratified by taking different treatment patterns. Finally, receiver-operating characteristic (ROC) curve analysis was performed to determine the diagnostic performance of miR-21 expression levels in distinguishing patients with AS from the healthy control subjects, and the area under the curve (AUC) value was also computed. All p values were calculated using 2-tailed statistical tests, and a p value < 0.05 was considered statistically significant. SAS 9.1 for Windows (SAS Inc.) was used for all analyses.

RESULTS

A total of 122 patients with AS and 122 healthy controls were recruited for this study, and their demographic and clinical characteristics are presented in Table 1. The proportion of male subjects was 72.1%. The average ages at recruitment of patients with AS and healthy controls were 37.5 ± 12.4 (SD) years and 37.5 ± 12.0 years, respectively. Because most continuous variables in our study showed skewed distribution, these variables were presented as median. Patients with AS had significantly higher leukocyte count than healthy controls ($p < 0.01$, Wilcoxon rank sum test). Among patients with AS, the age at symptom onset was 24.0 years and disease duration was 7.0 years. The proportion of HLA-B27–positive patients with AS was 90.2%. In addition, BASDAI, BASFI, BAS-G, and mSASSS were 4.2 cm, 1.3 cm, 4.8 cm, and 10.0, respectively.

Table 1. Demographic and clinical characteristics of patients with ankylosing spondylitis (AS) and healthy controls.

Variables	Patients with AS	Controls
No.	122	122
Sex (male)	88 (72.1%)	88 (72.1%)
Age at recruitment, yrs	37.5 ± 12.4[a]	37.5 ± 12.0
Laboratory test		
Leukocyte count ($10^3/\mu l$)	7.1 (1.0–15.7)[b,c]	6.2 (3.8–9.7)
HLA-B27+	110 (90.2%)	
ESR, mm/h	19.0 (12.0–34.0)[b]	
CRP, mg/dl	0.42 (0.12–1.20)[b]	
Clinical characteristics		
Age at symptom onset, yrs	24.0 (20.0–35.0)[b]	
Disease duration, yrs	7.0 (2.0–14.0)[b]	
Delayed diagnosis, yrs	0.0 (0.0–2.0)[b]	
Clinical syndromes		
Peripheral arthritis	36 (29.5%)	
Uveitis	29 (23.8%)	
Inflammatory bowel disease	4 (3.3%)	
BASDAI	4.2 (2.5–5.9)[b]	
BASFI	1.3 (0.2–3.6)[b]	
BAS-G	4.8 (2.0–6.5)[b]	
mSASSS	10.0 (4.0–20.0)[b]	

[a] Mean ± SD. [b] Data were presented as median (Q1–Q3). [c] $p < 0.01$; Wilcoxon rank sum test. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BAS-G: Bath Ankylosing Spondylitis Global; mSASSS: modified Stoke Ankylosing Spondylitis Spine Score.

The expression levels of miR-21, PDCD4 mRNA, and CTX of patients with AS and healthy controls are shown in Table 2. Compared to healthy controls, patients with AS had significantly higher levels of miR-21 ($p < 0.001$, Wilcoxon rank sum test), PDCD4 mRNA ($p < 0.001$), and CTX (0.31 vs 0.19 ng/ml, $p = 0.038$). Among the patients with AS, there were 17 without treatment, 102 who used NSAID (aceclofenac: 4 patients; celecoxib: 52 patients; etoricoxib: 23 patients; meloxicam: 23 patients), and 61 patients with DMARD use (methotrexate: 1 patient; SSZ: 60 patients; Table 3). However, no significant difference in the expression levels of miR-21, PDCD4 mRNA, and CTX level was observed in the different treatment groups of patients with AS.

Our study is focused on evaluating whether the corre-

Table 2. Expression levels of miR-21, PDCD4 mRNA, and CTX of patients with ankylosing spondylitis (AS) and controls. Data are presented as median (Q1–Q3). RNU6B and GAPDH were used as the endogenous controls for miR-21 and PDCD4 mRNA, respectively.

Variables	Patients with AS, n = 122	Controls, n = 122	p[a]	p[b]
miR-21 (2–ΔΔCT)	1.10 (0.27–3.29)	0.19 (0.07–0.60)	< 0.001	0.012
PDCD4 mRNA (2–ΔΔCT)	2.76 (1.67–3.85)	1.47 (1.00–2.25)	< 0.001	0.014
CTX, ng/ml	0.31 (0.14–0.55)	0.19 (0.16–0.38)	0.038	0.062

[a] Wilcoxon rank sum test was performed for comparison between patients with AS and controls. [b] Robust regression was performed for comparison between patients with AS and controls after adjusting for the effect of leukocyte count. miR-21: microRNA-21; PDCD4: programmed cell death 4; CTX: collagen cross-linked C-telopeptide.

Table 3. Expressions of miR-21, PDCD4 mRNA, CTX, and mSASSS in the different treatment patterns of patients with AS.

Treatment Pattern	n	miR-21 (2–ΔΔCT)	p	PDCD4 mRNA (2-ΔΔCT)	p	CTX (ng/ml)	p	mSASSS	p
Non-treatment	17	1.86 (0.57–4.83)	ref	2.29 (1.79–3.85)	ref	0.42 (0.14–0.85)	ref	10.0 (6.0–55.0)	ref
Treatment	105	1.09 (0.30–3.29)	0.441	2.80 (1.72–3.89)	0.711	0.31 (0.16–0.53)	0.838	10.0 (4.0–19.0)	0.217
NSAID use	102	1.08 (0.34–3.32)	0.474	2.81 (1.71–3.89)	0.771	0.30 (0.14–0.53)	0.948	10.0 (4.0–18.0)	0.184
Celecoxib	52	1.49 (0.25–3.05)	0.459	2.54 (1.52–3.78)	0.613	0.25 (0.10–0.50)	0.698	9.0 (5.0–18.0)	0.170
Etoricoxib	23	1.32 (0.40–8.15)	0.935	3.25 (2.19–4.58)	0.179	0.47 (0.22–0.67)	0.167	7.0 (0.0–21.0)	0.222
Meloxicam	23	0.90 (0.32–3.61)	0.465	2.50 (1.80–3.35)	0.807	0.30 (0.07–0.46)	0.833	9.5 (4.0–15.5)	0.461
DMARD use	61	1.32 (0.28–2.94)	0.448	2.77 (1.76–3.86)	0.656	0.33 (0.18–0.56)	0.695	12.0 (4.0–27.0)	0.348
Sulfasalazine	60	1.38 (0.28–3.11)	0.460	2.78 (1.75–3.87)	0.620	0.33 (0.18–0.57)	0.642	11.0 (4.0–24.0)	0.303

Data presented as median (Q1–Q3). The expressions of miR-21, PDCD4 mRNA, CTX, and mSASSS in the patients with AS with different treatment patterns compared to those with non-treatment by Wilcoxon rank sum test. Because too few subjects were treated with aceclofenac (n = 4) and methotrexate (n = 1), those subjects were not presented. mSASSS: modified Stoke Ankylosing Spondylitis Spine Score; miR-21: microRNA-21; PDCD4: programmed cell death 4; CTX: collagen cross-linked C-telopeptide; DMARD: disease-modifying antirheumatic drug; NSAID: nonsteroidal antiinflammatory drug.

lation of miR-21 and PDCD4 mRNA expressions changed in the patients with AS who received different treatments. As shown in Table 4, after adjusting for the effects of confounders, no significant correlation between miR-21 and PDCD4 mRNA levels was observed in patients with AS taking neither NSAID nor DMARD (r = –0.17, p = 0.59). Inversely, miR-21 expression was positively correlated with PDCD4 mRNA levels in patients with AS taking SSZ (r = 0.33, p = 0.01). Further, we assessed the correlation between miR-21 and CTX levels in the different treatment patterns of

Table 4. Correlations of miR-21 and PDCD4 mRNA expressions with CTX level, BAS indices, and laboratory tests in patients with AS (ankylosing spondylitis) with different treatments.

Variables	Non-treatment		Celecoxib		Etoricoxib		Meloxicam		Sulfasalazine	
	miR-21	PDCD4 mRNA	miR-21	PDCD4 mRNA	miR-21	PDCD4 mRNA	miR-21	PDCD4 mRNA	miR-21	PDCD4 mRNA
PDCD4 mRNA	r = –0.17	—	r = 0.16	—	r = 0.10	—	r = –0.04	—	r = 0.33*	—
CTX	r = 0.40	r = –0.15	r = 0.32*	r = 0.10	r = 0.54*	r = 0.07	r = 0.45*	r = –0.21	r = 0.44**	r = 0.10
mSASSS	r = –0.26	r = –0.41	r = –0.09	r = –0.04	r = –0.13	r = 0.27	r = 0.27	r = –0.15	r = –0.11	r = –0.18
BASDAI	r = 0.14	r = 0.32	r = 0.06	r = –0.0007	r = 0.07	r = –0.13	r = –0.25	r = –0.36	r = 0.01	r = –0.01
BASFI	r = 0.21	r = –0.49	r = 0.02	r = –0.06	r = –0.13	r = –0.11	r = –0.26	r = –0.15	r = –0.06	r = –0.08
BAS-G	r = 0.12	r = 0.25	r = 0.14	r = 0.03	r = 0.17	r = –0.29	r = –0.15	r = –0.24	r = 0.14	r = –0.13
ESR	r = 0.18	r = –0.76**	r = –0.16	r = 0.09	r = 0.15	r = 0.04	r = 0.18	r = –0.35	r = –0.06	r = –0.08
CRP	r = 0.23	r = –0.50	r = –0.07	r = 0.01	r = 0.02	r = –0.18	r = 0.17	r = –0.26	r = –0.03	r = –0.04

Adjusted for the effects of age, sex, and disease duration. * $0.01 \le p \le 0.05$. ** $p < 0.01$. mSASSS: modified Stoke Ankylosing Spondylitis Spine Score; miR-21: microRNA-21; PDCD4: programmed cell death 4; CTX: collagen cross-linked C-telopeptide; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BAS-G: Bath Ankylosing Spondylitis Global; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

patients with AS. A significantly positive correlation of miR-21 and CTX levels was observed in patients with AS taking celecoxib (r = 0.32, p = 0.03), etoricoxib (r = 0.54, p = 0.01), meloxicam (r = 0.45, p = 0.05), and SSZ (r = 0.44, p < 0.01). In addition, there was a significantly negative correlation between PDCD4 mRNA and ESR levels in patients with untreated AS (r = –0.76, p < 0.01). However, we could not observe the significant correlations of miR-21, PDCD4 mRNA expressions with BAS index, and mSASSS in our patients with AS with different drug treatments.

Because low bone density has been demonstrated in patients with AS, particularly in the early stage of the disease[4], we further evaluated the correlation of miR-21 and CTX levels in patients with AS stratified by disease duration. The median of disease duration of our patients with AS was 7.0 years (Table 1), thus 7.0 years of disease duration was used as a cutoff point. Interestingly, miR-21 expression was more obviously correlated with CTX levels in patients with AS with disease duration < 7.0 years (r = 0.36, p = 0.004, Figure 1A). Previous evidence indicated the disease activity marker BASDAI might affect bone mineral density in patients with AS[23]. In our present study, patients with active disease were defined as those with a BASDAI score ≥ 4[24,25]. We further evaluated the correlation of miR-21 and CTX levels in patients with active and inactive AS. As expected, a significantly positive correlation of miR-21 and CTX levels was observed in patients with active AS (r = 0.42, p = 0.001, Figure 1C).

We also performed ROC analysis to determine the diagnostic performance of miR-21 expression levels in distinguishing patients with AS from the healthy control subjects. The expression levels of miR-21 in whole blood yielded an AUC value of 0.757 (95% CI 0.698–0.817) in distinguishing patients with AS from controls (Figure 2). When patients with drug treatment were excluded, AUC value was elevated to 0.789 (95% CI 0.647–0.877).

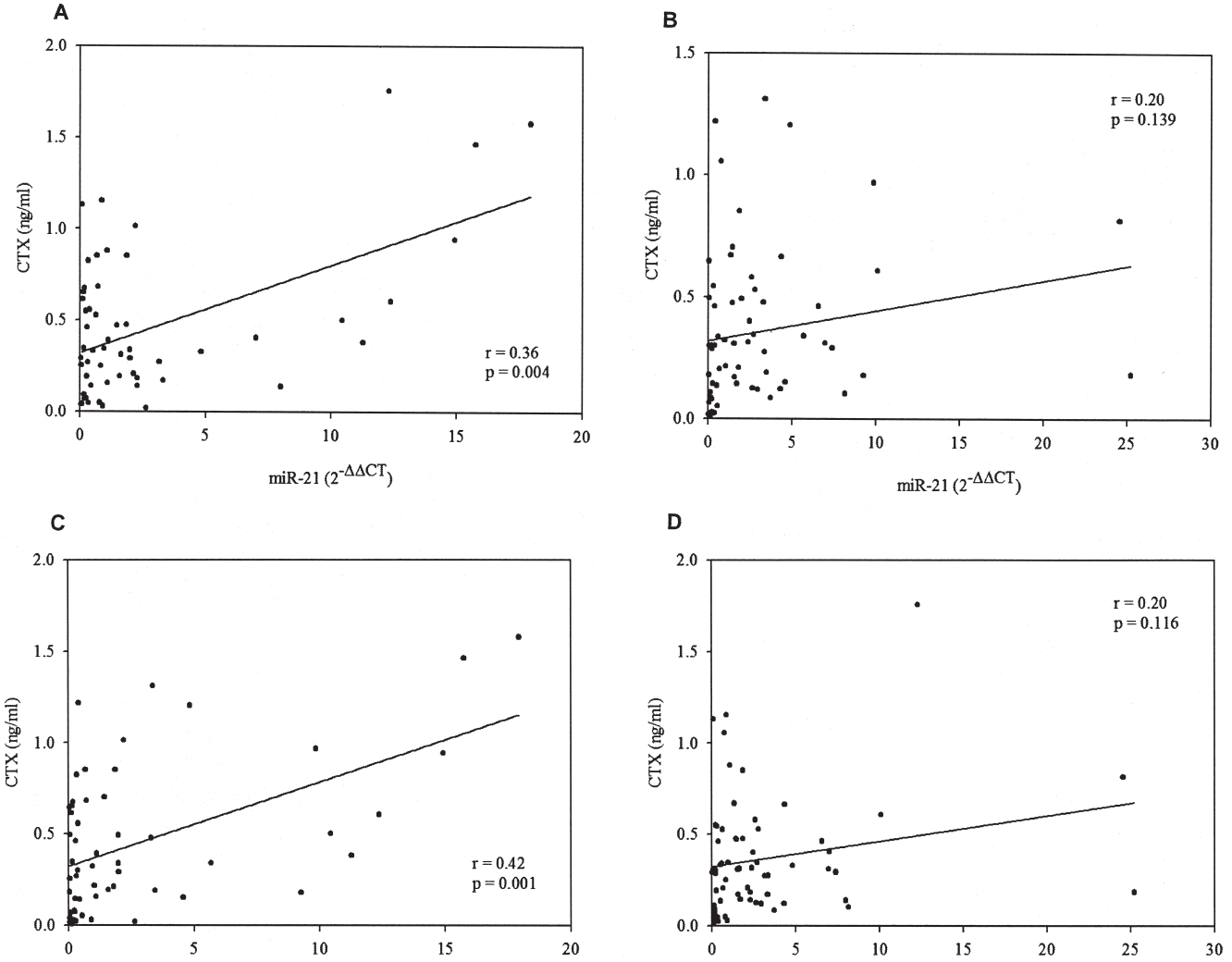

Figure 1. Correlation between miR-21 and CTX levels. A. Correlation in AS patients with disease duration < 7.0 years (n = 58). B. Correlation in AS patients with disease duration ≥ 7 years (n = 64). C. Correlation in active AS patients (n = 66). D. Correlation in patients with inactive AS (n = 56). RNU6B was used as the endogenous control for miR-21. miR-21: microRNA-21; CTX: collagen cross-linked C-telopeptide; AS: ankylosing spondylitis.

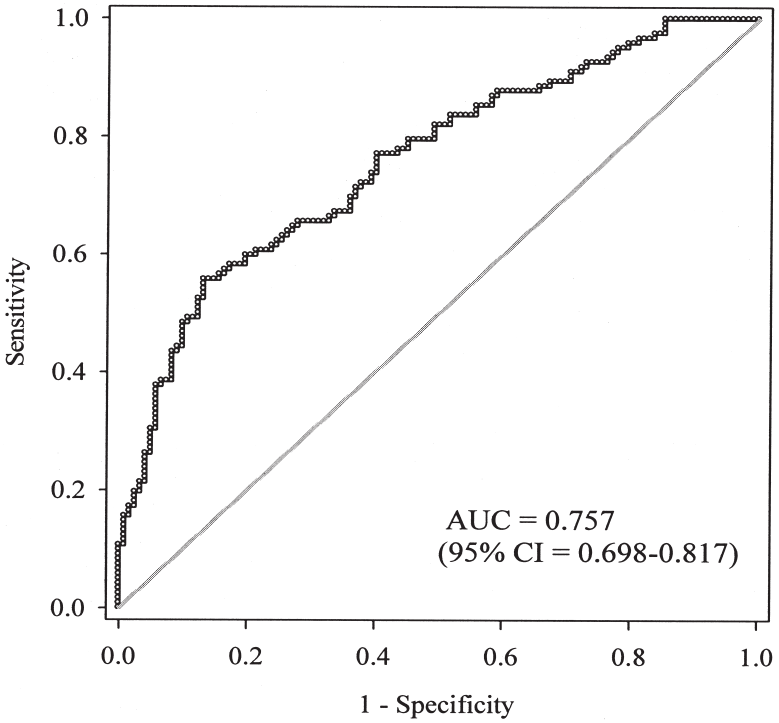

Figure 2. Receiver-operating characteristics curve analysis using miR-21 expression in whole blood for distinguishing patients with AS from control subjects. The area under the curve (AUC) value and 95% CI were computed. miR-21: microRNA-21.

DISCUSSION

Previously, miR-21 has been reported to be highly expressed in osteoclastogenesis[26]. Our study, to our knowledge, was the first to observe that the expression of miR-21 in patients with AS was significantly greater than in healthy controls. Interestingly, our patients with AS also had increased levels of serum CTX compared to healthy controls, and those with lower miR-21 level more frequently had lower CTX levels. However, no correlation of miR-21 expression and mSASSS was observed in our patients with AS. Taken together, our findings suggest the expression of miR-21 may be a potential biomarker in the bone erosion of patients with AS, but might not correlate with bone formation.

It has been proposed that PDCD4 may be one of the repressor proteins for osteoclastogenesis[9]. Evidence also showed PDCD4 is the target gene of miR-21[27]. However, our results unexpectedly showed that the expression of PDCD4 mRNA in patients with AS was higher than that in healthy controls. This might be because the PDCD4 expression is modified by certain drugs in our patients with AS. It has been suggested that NSAID could reduce the signs and symptoms of AS[28]. SSZ, a well-established DMARD, is also recommended for the treatment of spondyloarthritis, such as AS. The use of SSZ in patients with AS also brought about marked improvement in inflammatory indices, including ESR[29]. In our study, the negative correlation of PDCD4 expression and ESR could be observed only in patients with AS who were taking neither NSAID nor DMARD. Such results reflected that inflammation might suppress PDCD4 expression[30], and the use of NSAID or DMARD might influence inflammation and PDCD4 expression in patients with AS. In addition, our results observed a positive correlation between miR-21 and PDCD4 mRNA expression in patients with AS taking SSZ. A previous study also indicated that the expression of caspases 3 and 8 in the synovial tissue of patients with RA were significantly increased after DMARD treatment[31]. In our patients with AS, those taking SSZ had increased PDCD4 mRNA expression compared to those not taking NSAID or DMARD (median of 2-ΔΔCT: 2.78 vs 2.29, Table 3). Therefore, the use of SSZ is likely to induce the expression of apoptosis-associated molecules such as PDCD4. However, this result had limited inferential capability, because the small sample size limited our statistical power. The correlation between miR-21 and PDCD4 expression should be re-examined in a larger study according to the use of specific drugs.

In our study, the patients with AS also had an increased level of serum CTX compared to healthy controls. A previous study found a higher level of bone erosion in younger AS patients[4]. In contrast, older patients with inactive disease were more likely to have lower levels of

bone erosion. Therefore, the bone loss of patients with AS might be associated with disease duration. Further, our study observed miR-21 expression was positively correlated with CTX levels only in patients with AS with a disease duration < 7 years. In addition, miR-21 expression was not significantly correlated with CTX levels in our healthy controls. Evidence also indicated that disease activity in AS plays a critical role in the pathogenesis of bone loss[32], and this role might be controlled by miRNA and cytokines that regulate inflammation and bone turnover[33,34]. As expected, our results found a significantly positive correlation of miR-21 and CTX levels in active patients with AS (BASDAI ≥ 4). A transgenic mice model of allergic asthma found inflammation could induce the expression of miR-21[35]. Crucially, inflammation in patients with AS might have the potential to induce osteoclastogenesis[4] and has been associated with disease activity[36]. Similarly, our patients with AS who had shorter disease duration also had increased expression of miR-21 (median of 2-ΔΔCT: < 7.0 years, 1.38 vs ≥ 7.0 years, 1.09), although it was not statistically significant. Therefore, miR-21 might only play a prominent role during inflammation. Even so, it is possible that patients with AS with short disease duration have an elevated expression of miR-21 to induce bone erosion, especially in those with higher disease activity associated with inflammation.

In our study, the expression levels of miR-21 in whole blood yielded an AUC value around 0.8 in distinguishing patients with AS from controls. This discrimination is acceptable but not very good. Thus, re-evaluations are required to determine the usefulness of miR-21 as a disease biomarker of AS. In our study, the expression of whole blood miR-21 and of PDCD4 mRNA were measured by RT-qPCR done in triplicate, and the difference of the Ct values in triplicate had to be < 0.5. Therefore, our RNA expression data had good reliability. However, it has been noted that miRNA concentrations in different blood compositions are varied[37]. Our results also observed that patients with AS had significantly higher leukocyte count than healthy controls. The difference of miR-21 expression was still significant between patients with AS and healthy controls after adjustment for the effect of leukocyte count. In our study, serum CTX was chosen for assessing bone resorption because of its higher specificity and lower variability as compared to other tests currently available[15], and CTX is a well-established biomarker for phenotype osteoporosis. Chronic spinal changes in our patients with AS were also quantified by the mSASSS[20], but no correlation of miR-21 and PDCD4 mRNA expressions with mSASSS was observed in our patients with AS. However, bone formation rather than bone erosion might be developed in the late disease progress of AS[38]. Thus, the effect of bone formation-associated molecules should be evaluated in further studies in patients with AS with longer disease duration. In our study, no functional experiments were performed to determine whether miR-21 actually acts as a relevant molecule in pathological osteoclastogenesis in AS. Further functional studies are required to test our findings. Referral bias was also possible, because our data were collected from a single medical center. Finally, larger cohort studies are necessary to provide further evidence regarding our findings.

Our current findings suggest that the expression of miR-21 might have a role in the development of AS.

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