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ORIGINAL ARTICLE

MiR-29a and Messenger RNA Expression of Bone Turnover Markers in Canonical Wnt Pathway in Patients with Ankylosing Spondylitis

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SUMMARY

Background: **Recent studies showed that the canonical Wnt pathway and miR-29a play important roles in the pathogenesis of bone formation. We studied the levels of miR-29a and messenger RNA (mRNA) of bone turnover markers in the canonical Wnt pathway in ankylosing spondylitis (AS).**
Methods: **The levels of miR-29a and mRNA of bone turnover markers in canonical Wnt pathway from peripheral blood mononuclear cells were determined by real-time quantitative polymerase chain reaction in 38 patients with AS and 32 healthy controls.**
Correlation analysis was conducted between the levels of miR-29a and mRNA and clinical measurements using Spearman's correlation test.
Results: **Compared to healthy controls, the levels of miR-29a, Dickkopf (DKK)-1, β-catenin and Runx2 mRNA were significantly higher in AS patients ($p < 0.05$). In contrast, the levels of Gsk-3β mRNA was significantly lower in AS patients than that in healthy controls ($p < 0.05$). Gsk-3β mRNA was positively correlated with β-catenin mRNA expression ($p < 0.05$) and no other correlation was observed between any other markers ($p > 0.05$). Only DKK-1 mRNA expression was negatively correlated with disease course ($p < 0.05$) and no other correlation was observed between markers and clinical measurements ($p > 0.05$).**
Conclusions: **The osteoblastic marker miR-29a and downstream mRNA of canonical Wnt signaling was upregulated in AS, suggesting their possible role in new bone formation in AS.**
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INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disorder characterized by new bone formation that progressively leads to ankylosis and functional disability. The canonical Wnt pathway might play a critical contributing role in bone fusion in AS [1]. Recent clinical observations and animal studies demonstrate that Wnt signaling proteins and natural Wnt inhibitors, such as DKK1 and sclerostin, are likely to play important roles in the process of ankylosis in the disease

[2]. DKK-1 is a well-known negative regulator of the canonical Wnt pathway and the down regulation of DKK-1 might lead to the activation of the signaling pathway [3]. In mice transgenic for tumor necrosis factor (TNFtg mice) which develop bilateral sacroiliitis, blockade of DKK-1 induces fusion of sacroiliac joints [4].

Both canonical Wnt signaling and miR-29 promote osteoblast differentiation through a variety of mechanisms. MiR-29a positively regulated osteoblast differentiation mainly through targeting the canonical Wnt pathway [5]. DKK-1 is the direct target of miR-29a and miR-29a negatively regulates endogenous levels of DKK-1 in ex vivo studies [6]. Our previous study [7] demonstrated elevated miR-29a level in AS patients and correlation with disease duration as well as pathologic progression as evaluated by the modified Stoke's Ankylosing Spondylitis Spine Score (mSASSS) index [8]. A recent study determined lower miR-29a expression in PBMCs from active AS patients and dramatic upregulation after etanercept treatment without correlation with erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [9], which was hard to explain. Discrepancy of miR-29a expression might be due to distinct patient enrollment in the two studies, while correlation between clinical indexes needs further investigation.

In this study, we aim to investigate the miR-29a level and its correlation with mRNA expression of bone turnover markers in the canonical Wnt pathway and clinical parameters, in order to reveal the bone formation mechanism of miR-29a participation in the signaling pathway.

MATERIALS AND METHODS

Subject recruitment and sample preparation

All patients were diagnosed with AS according to the modified New York criteria [10]. The study was approved by the Ethics Committee. All participating subjects gave written consent according to the Declaration of Helsinki. Laboratory tests including ESR, CRP, and human leukocyte antigen (HLA)-B27 were conducted. Clinical data including BASDAI, Bath Ankylosing Spondylitis Functional Index (BASFI), and Ankylosing Spondylitis Disease Activity Score (ASDAS) were evaluated. All patients underwent an X-ray study of the cervical and lumbar spine to calculate the mSASSS index. PBMCs were prepared according to the manufacturer's instruction.

Measurement of miRNA levels

cDNA was reversed from total RNA including miRNAs using the TaqMan MicroRNA kit. The resulting cDNA was stored at -80°C. The expression of miR-29a was quantified by real-time quantitative polymerase chain reaction (RT-QPCR) (Applied Biosystems). For RT-QPCR, 10 μL universal master mix, 1 μL primer, 1 μL cDNA, and 8 μL H_2O were mixed to make a 20 μL reaction volume. RT-QPCR was performed at 95°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds and 55°C for 1 minute. Each sample was run in triplicate. U6 small nuclear RNA was quantified as a control to normalize differences in total RNA levels. The ΔΔCt method [11] was used in the analysis of PCR data [ΔCt = mean Ct (microRNA of interest) - mean Ct (U6)].

Messenger RNA extraction and reverse transcription

Total RNA was extracted from PBMCs using the Trizol method. The purity of RNA was confirmed by the relative absorbance ratio at 260/280 nm using a spectrometer.

RNA was reversed to cDNA by the Transcriptor First Strand cDNA kit. For real-time quantitative PCR, 10 μL 2 x SYBR Green I Master mix, 0.4 μL primer, 1 μL ROX, 2 μL cDNA, and 6 μL H_2O were mixed to make a 20 μL reaction volume. Each sample was run in triplicate. Real-time quantitative PCR was performed at 95°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds, 55 - 60°C for 15 seconds and 72°C for 30 seconds.

Ct value of β-actin was used as an internal control and compared with that of the target gene. The ΔΔCt method for relative quantity was used as previously described to calculate the differences of expression level for each target gene among samples.

Statistical analysis

Statistical analysis was performed by SPSS for Windows software version 13.0. All the results were presented in mean ± SD. Spearman's rank correlation was used to test the association between gene expression levels and clinical measurements. A p-value < 0.05 was considered statistically significant. All probabilities were 2-tailed.

RESULTS

In total, 38 AS patients and 32 healthy controls were enrolled in our study. The distribution of age and gender was similar between AS patients and healthy controls ($p > 0.05$). The mean disease duration was 9.25 ± 7.93 years. The mean mSASSS was 13.74 ± 15.26. ESR and CRP were 21.47 ± 21.29 mm/h and 12.86 ± 14.35 mg/L, respectively. The mean BASDAI, BASFI, and ASDAS scores were 3.79 ± 1.91, 41.13 ± 21.02, and 1.94 ± 1.00, respectively.

The levels of miR-29a was significantly higher in AS patients (6.92 ± 5.60) than that in healthy controls (4.38 ± 4.04) ($p < 0.05$). AS patients had significantly higher levels of DKK-1 (1.41 ± 1.38 vs. 0.45 ± 0.44; $p < 0.001$), β-catenin (0.22 ± 0.20 vs. 0.05 ± 0.04; $p < 0.001$), and Runx2 (2.62 ± 2.27 vs. 0.67 ± 0.40; $p < 0.001$) mRNA expression compared with healthy

Table 1. MiR-29a and mRNA expression bone turnover markers in canonical Wnt signaling in AS patients and healthy controls.

	AS (n = 38)	Healthy control (n = 32)	p-value
miR-29a	6.92 ± 5.60	4.38 ± 4.04	0.036
DKK-1	1.41 ± 1.38	0.45 ± 0.44	0.0002
Gsk-3β	3.54 ± 3.51	6.02 ± 5.85	0.032
β-catenin	0.22 ± 0.20	0.05 ± 0.04	1.04×10^{-5}
Runx2	2.62 ± 2.27	0.67 ± 0.40	9.06×10^{-6}

Table 2. Correlations between miR-29a and mRNA expression of bone turnover markers in canonical Wnt pathway in AS patients.

	miR-29a	DKK-1	Gsk-3β	β-catenin	Runx2
miR-29a		r = -0.101 p = 0.545	r = -0.009 p = 0.958	r = -0.057 p = 0.735	r = -0.197 p = 0.237
DKK-1	r = -0.101 p = 0.545		r = 0.231 p = 0.162	r = -0.263 p = 0.111	r = 0.029 p = 0.863
Gsk-3β	r = -0.009 p = 0.958	r = 0.231 p = 0.162		r = 0.522 * p = 0.001	r = 0.243 p = 0.141
β-catenin	r = -0.057 p = 0.735	r = -0.263 p = 0.111	r = 0.522 * p = 0.001		r = 0.250 p = 0.131
Runx2	r = -0.197 p = 0.237	r = 0.029 p = 0.863	r = 0.243 p = 0.141	r = 0.250 p = 0.131	

*** Gsk-3β was positively correlated with β-catenin ($p < 0.05$).**

Table 3. Correlations between miR-29a and mRNA expression of bone turnover markers and clinical parameters in patients with AS.

	ESR (mm/h)	CRP (mg/L)	Disease course (years)	mSASSS	BASDAI	BASFI	ASDAS
miR-29a	r = -0.080 p = 0.600	r = 0.069 p = 0.679	r = -0.033 p = 0.843	r = -0.152 p = 0.364	r = 0.081 p = 0.629	r = -0.001 p = 0.995	r = 0.049 p = 0.772
Dkk1	r = 0.017 p = 0.919	r = -0.014 p = 0.931	r = -0.395 * p = 0.014	r = -0.078 p = 0.642	r = -0.084 p = 0.617	r = -0.105 p = 0.530	r = -0.026 p = 0.877
Gsk-3β	r = -0.109 p = 0.514	r = -0.100 p = 0.551	r = -0.027 p = 0.874	r = -0.042 p = 0.802	r = -0.039 p = 0.815	r = 0.071 p = 0.672	r = -0.016 p = 0.924
β-catenin	r = -0.186 p = 0.263	r = -0.117 p = 0.485	r = -0.003 p = 0.984	r = 0.086 p = 0.609	r = -0.029 p = 0.864	r = 0.015 p = 0.928	r = -0.087 p = 0.603
Runx2	r = 0.004 p = 0.980	r = -0.171 p = 0.305	r = -0.137 p = 0.412	r = -0.089 p = 0.597	r = -0.082 p = 0.624	r = -0.056 p = 0.740	r = -0.154 p = 0.356

Results are shown as the correlation coefficients and p-values, calculated using Spearman's correlation test.
*** DKK-1 mRNA expression was negatively correlated with disease course ($p < 0.05$).**

controls. In contrast, the levels of Gsk-3β mRNA was significantly lower in AS patients (3.54 ± 3.51) than that in healthy controls (6.02 ± 5.85) ($p < 0.05$). (see Table 1, Figure 1a - e).

In the correlation analysis between miR-29a and mRNA expression of bone turnover markers in the canonical Wnt pathway in AS patients, only Gsk-3β was positively correlated with β-catenin ($p < 0.05$) and no other cor-

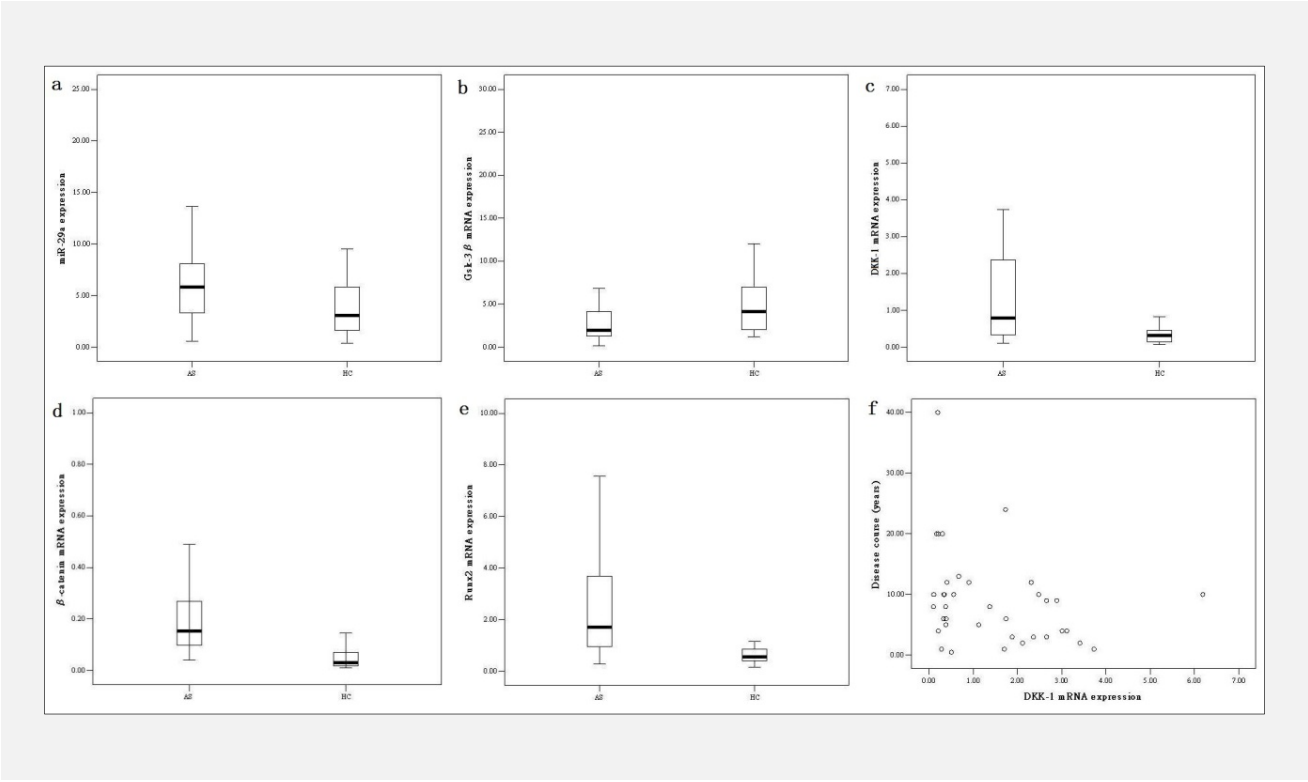

Figure 1. Comparison of miR-29a (a), GSK-3β (b), DKK-1 (c), β-cateinin (d) and Runx2 (e) mRNA expression in AS patients and healthy controls. Positive correlation between DKK-1 mRNA expression and disease course (f) in AS patients.

relation was observed between any other markers ($p > 0.05$) (see Table 2).

In the correlation analysis between miR-29a and mRNA expression of bone turnover markers and clinical parameters in patients with AS, only DKK-1 mRNA expression was negatively correlated with disease course ($p < 0.05$) (Figure 1f) and no other correlation was observed between markers and clinical measurements ($p > 0.05$) (see Table 3).

DISCUSSION

Our study demonstrated that miR-29a was highly expressed in AS, suggesting the potential osteoblastic role in the mechanism of bone formation in AS. The accompanied low expression of GSK3β and high expression of both β-catenin and Runx2 also indicate the activation of Wnt/β-catenin signaling in AS. In fact, it has already been reported that Runx2 mRNA expression was upregulated in AS compared with RA [12], which is consistent with the discovery in our study. To our knowledge, we are the first to detect GSK3β and β-catenin mRNA expression in AS.

Paradoxically, DKK-1 was highly expressed in AS. Although this cannot perfectly explain the miR-29a high level and activation of Wnt signaling in AS, this was consistent with the serum expression of DKK-1 in some previous studies [13-16]. Nevertheless, the serum expression of DKK-1 in AS has been controversial, with some other studies [17-20] reporting decreased or similar levels of DKK-1 in AS compared with healthy controls. DKK-1 might have a compensatory effect in preventing the formation of syndesphocyte [21], or was dysfunctional [13,22] in AS. A negative feedback mechanism of DKK-1 that limits Wnt-driven bone formation was proven in human liver tumor [23], zebrafish [24], rodents, and non-human primates [25], which might explain the up-regulation of DKK-1 in our study. miR-29a targets directly on the 3'UTR of both DKK-1 and GSK3β as verified by putative binding site analysis (TargetScan and RNAhybrid) and dual luciferase reporter gene system. Besides, TCF/LEF transcriptional activity was greatly increased by overexpression of miR-29a or depletion of DKK-1 or GSK3β, suggesting increased nuclear β-catenin accumulation and activation of the canonical Wnt signaling [26]. GSK3β negative regulation by miR-29a might outweigh the role of DKK-1 in our set of individuals.

miR-29a is a key player of osteogenic differentiation, which interacts with canonical Wnt signaling by targeting many putative targets [27]. Nonetheless, elevated mir-29a was not in correlation with the activation of Wnt components in our study. Linear association was

not noted in between miR-29a and Wnt signaling, suggesting that the complicated microenvironment might be involved in the mechanism of bone formation in AS. It was also hypothesized that miR-29a positively regulates osteoblast differentiation by controlling the expression of osteonectin and likely other targets in canonical Wnt signaling. miR-29a modulation of osteoclast differentiation was linked to the osteoclast regulatory factor RANKL or to OPG expression as well [28]. Besides miR-29a, other microRNAs can also have synergic effects on the activation of the pathway. Crosstalk among different signaling pathways might also contribute to the bone formation in AS.

The important roles of β-catenin signaling in knee joint [29], intervertebral disc [30], and temporomandibular joint (TMJ) [31] tissues were demonstrated in previous studies. The β-catenin protein was up-regulated in disc tissues from patients with disc degeneration. Activation of β-catenin signaling in articular chondrocytes in adult mice leads to the premature chondrocyte differentiation and the development of an OA-like phenotype. Overexpressing β-catenin in TMJ cartilage leads to defects assembling an OA-like phenotype. Gsk-3β was positively correlated with β-catenin, suggesting a tight relationship between these two components in the canonical Wnt pathway. On the contrary, other components were not correlated with each other in the pathway. The most probable explanation would be a complex translation procedure from mRNA to protein leading to mismatched expression in downstream sections. These components might also take effect in other pathways besides the canonical Wnt pathway.

MiR-29a was not correlated with ESR, CRP or disease activity indexes, indicating that inflammation and new bone formation were separate processes. This phenomenon was supportive from the fact that new bone formation was not necessarily reversed by TNF inhibitor treatment [32].

Our former study discovered that prolonged disease duration was accompanied by progression in new bone formation as revealed by mSASSS and elevated miR-29a expression [7], while this was not the case in the present study. Elevated miR-29a expression was not correlated with either disease course or mSASSS, indicating distinct mechanism in different patient subsets concerning this heterogeneous disease.

MiR-29a might be a useful marker contributing for screening individuals at high risk for new bone formation with reasonable grouping.

miR-29a is a well-known osteogenic factor and its signaling protects against glucocorticoid-induced disturbance of Wnt and DKK-1 actions and improved osteoblast differentiation and mineral acquisition [33]. The study also demonstrated that canonical Wnt signaling, which is increased during osteoblastic differentiation, induces expression of miR-29a [34]. This positive feedback regulatory circuit [35] provides additional insight into how microRNAs interact with signaling molecules during osteoblast differentiation and might partially explain how miR-29a is upregulated in patients with AS.

CONCLUSION

Elevated miR-29a and activation of canonical Wnt signaling was noticed in AS, which might partially explain the new bone formation in AS.

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Declaration of Interest:
The authors report no competing financial interests.

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