

lncRNA MEG3 is downregulated in ankylosing spondylitis and associated with disease activity, hospitalization time and disease duration

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Abstract. Long non-coding (lnc)RNA maternally expressed gene 3 (MEG3) has been proved to participate in osteoporosis, which features inverse pathological changes to those associated with spondylosis. The present study aimed to investigate the involvement of lncRNA MEG3 in ankylosing spondylitis. Blood and open sacroiliac joint biopsies were obtained from ankylosing spondylitis patients and healthy controls, and the expression of MEG3 in those tissues was detected by reverse-transcription-quantitative polymerase chain reaction analysis. Disease activity was evaluated according to the Ankylosing Spondylitis Disease Activity Score established by the International Association of Ankylosing Spondylitis. The diagnostic value of MEG3 expression for ankylosing spondylitis was evaluated by receiver operating characteristic curve analysis. The correlation between MEG3 expression and disease activity was assessed using Pearson correlation analysis. Furthermore, according to the median expression level of MEG3, patients were divided into high-level and low-level groups. The hospitalization time and re-hospitalization rate within 2 years after discharge were compared between these two groups and differences in clinicopathological parameters between the two groups were analyzed using the chi-square test. The results indicated that MEG3 was downregulated in ankylosing spondylitis patients compared with that in healthy controls. Furthermore, MEG3 expression levels may be used to effectively distinguish ankylosing spondylitis patients from healthy controls. The serum levels of MEG3 were not associated with the patients' age, sex or alcohol/tobacco consumption, but closely correlated with disease activity and

disease duration. In addition, patients with higher expression levels of MEG3 had a shorter hospitalization time and a lower re-hospitalization rate within 2 years after discharge. It was concluded that lncRNA MEG3 is downregulated in ankylosing spondylitis patients and is associated with disease activity, time of hospitalization and disease duration.

Introduction

Ankylosing spondylitis is a type of arthritis characterized by the long-term inflammation of the joints of the spine (1). This disease mainly affects spine joints, while other joints, including hips or shoulders, may also be affected in certain cases (1). Stiffness of the affected joints becomes worse with the development of ankylosing spondylitis, leading to impaired back mobility and reduced life quality (2,3). Ankylosing spondylitis mainly affects individuals aged 20-30 years and the early diagnosis rate is low (4). Although human leukocyte antigen (HLA) B27 subtypes (B*2701-2759) and bacteria (e.g. *Enterobacter*) have been proved to be correlated with the progression of ankylosing spondylitis, their diagnostic value for this disease is low (4). Therefore, identification of novel effective and reliable diagnostic markers is urgently required to improve the survival of patients with ankylosing spondylitis.

Long non-coding (lnc)RNAs are a class of RNA transcripts comprising >200 nucleotides with no protein coding capacity but the ability to post-transcriptionally regulate the expression of certain genes (5). lncRNAs have important functions in normal physiological processes and pathological changes in the human body (6,7). The role of the lncRNA maternally expressed gene 3 (MEG3) as a tumor suppressor has been extensively characterized in different types of cancer (8).

A recent study proved that MEG3 may promote the development of osteoporosis (9), which has mechanistic similarities with ankylosing spondylitis (10), while its possible role in ankylosing spondylitis has remained elusive. Therefore, the present study investigated the involvement of MEG3 in ankylosing spondylitis, revealing that lncRNA MEG3 was downregulated in ankylosing spondylitis and is associated with disease activity, disease duration, hospitalization time and re-hospitalization rate within 2 years after discharge.

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The present study provided references for the diagnosis and prognosis of ankylosing spondylitis.

Materials and methods

Subjects. A total of 172 patients with ankylosing spondylitis who presented at Yongchuan Hospital of Chongqing Medical University (Chongqing, China) between January 2013 and January 2015 were included in the present study. All patients were diagnosed according to the New York criteria established in 1984 (11). Those patients included 98 males and 74 females, and their age ranged from 10 to 45 years, with a mean age of 25.6 ± 5.4 years. All patients were diagnosed and treated for the first time. Disease activity was evaluated based on the Ankylosing Spondylitis Disease Activity Score (ASDAS) (12), which was interpreted as follows: <1.3 , inactive disease; $1.3-2.1$, moderate disease activity; $2.1-3.5$, high disease activity; and >3.5 very high disease activity. Patients transferred to other hospitals during treatment were not included. All patients were followed up for 5 years and re-hospitalization within 2 years after discharge was recorded. At the same time, 98 control subjects with normal physiological conditions were also included to serve as a healthy control group. The control group included 55 males and 43 females, and the age ranged from 14 to 42 years, with a mean age of 26.1 ± 6.3 years. No significant differences in age and sex were identified between the patient and the control group.

Specimen collection. Blood (~ 15 ml) was obtained from the elbow vein of each of the patients and healthy control subjects on the day of admission. Blood was kept at room temperature for 1.5 h and then centrifuged at $800 \times g$ for 20 min at room temperature to collect the serum. Open sacroiliac joint biopsies were collected from 42 patients and 36 healthy controls. All specimens were stored in liquid nitrogen prior to analysis. Open sacroiliac joint biopsies were performed on healthy controls to detect potential lesions, but those potential lesions were not found and no medical conditions were observed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Biopsies were ground in liquid nitrogen, followed by addition of TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to extract total RNA. Serum samples were directly mixed with TRIzol reagent to extract total RNA. Total RNA samples were then used as template to synthesize complementary (c)DNA through RT using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 25°C for 5 min, 55°C for 20 min and 80°C for 20 min. The PCR reaction system was prepared using the SYBRTM Green PCR Master mix (cat. no. LS4309155; Applied BiosystemsTM; Thermo Fisher Scientific, Inc.) and cDNA samples. The sequences of the primers used for PCR were as follows: Human lncRNA-MEG3 forward, 5'-CTGCCCATCTACACCTCACG-3' and reverse, 5'-CTCTCCGCCGTCTGCGCTAGGCGCT-3'; human β -actin forward, 5'-GACCTCTATGCCAACACAGT-3' and reverse, 5'-AGTACTTGCGCTCAGGAGGA-3'. PCR analyses were performed on the CFX384 TouchTM Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following thermocycling

conditions: 95°C for 45 sec, followed by 40 cycles of 95°C for 10 sec and 52°C for 35 sec according to manufacturer's protocol. The quantification of mRNA expression was performed using the $2^{-\Delta\Delta\text{Cq}}$ method (13). The expression levels of lncRNA-MEG3 relative to those of the endogenous control β -actin were determined.

Statistical analysis. SPSS version 19.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Receiver operating characteristics (ROC) curve analysis was performed using default parameters. Comparisons of measurement data expressed as the mean \pm standard deviation between two groups were performed by using the unpaired t-test. Count data were compared by Chi-squared test. Correlation analyses were performed by a Pearson correlation analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of expression levels of lncRNA-MEG3 in serum and open sacroiliac joint biopsies between ankylosing spondylitis patients and healthy controls. RT-qPCR was performed to detect the expression of lncRNA-MEG3 in serum and open sacroiliac joint biopsies collected from ankylosing spondylitis patients and healthy controls. As presented in Fig. 1, a significantly downregulated MEG3 expression in the serum (Fig. 1A) and open sacroiliac joint biopsies (Fig. 1B) of ankylosing spondylitis patients compared with that in healthy controls was identified. These results suggest that downregulation of lncRNA-MEG3 expression is likely to be involved in the pathogenesis of ankylosing spondylitis.

Diagnostic value of lncRNA-MEG3 expression in serum and open sacroiliac joint biopsies for ankylosing spondylitis. ROC curve analysis was performed to evaluate the diagnostic values of lncRNA-MEG3 expression in serum and open sacroiliac joint biopsies for ankylosing spondylitis. The ROC curve for serum lncRNA-MEG3 is presented in Fig. 2A. The area under the curve (AUC) of the ROC for the use of serum lncRNA-MEG3 in the diagnosis of ankylosing spondylitis was 0.7484 with a 95% confidence interval (CI) of 0.6950-0.8017 ($P < 0.0001$). The ROC curve for lncRNA-MEG3 in open sacroiliac joint biopsies is presented in Fig. 2B. The AUC of the ROC for the use of lncRNA-MEG3 expression in open sacroiliac joint biopsies in the diagnosis of ankylosing spondylitis was 0.8862 with a 95% CI of 0.8163-0.9562 ($P < 0.0001$).

Correlation between serum circulating MEG3 levels with clinicopathological characteristics of ankylosing spondylitis patients. A linear correlation analysis was performed with MEG3 expression as 'X' values and ASDAS scores as 'Y' values. The results suggested that the levels of MEG3 in the serum ($R^2 = 0.6198$, $P < 0.0001$; Fig. 3A) and open sacroiliac joint biopsies ($R^2 = 0.6222$, $P < 0.0001$; Fig. 3B) were significantly positively correlated with ASDAS. According to the medium expression levels of MEG3, patients were divided into high-level and low-level groups ($n = 86$). The differences between these high-level and low-level groups regarding the general clinicopathological data were further analyzed by using the chi-square test. As presented in Tables I and II,

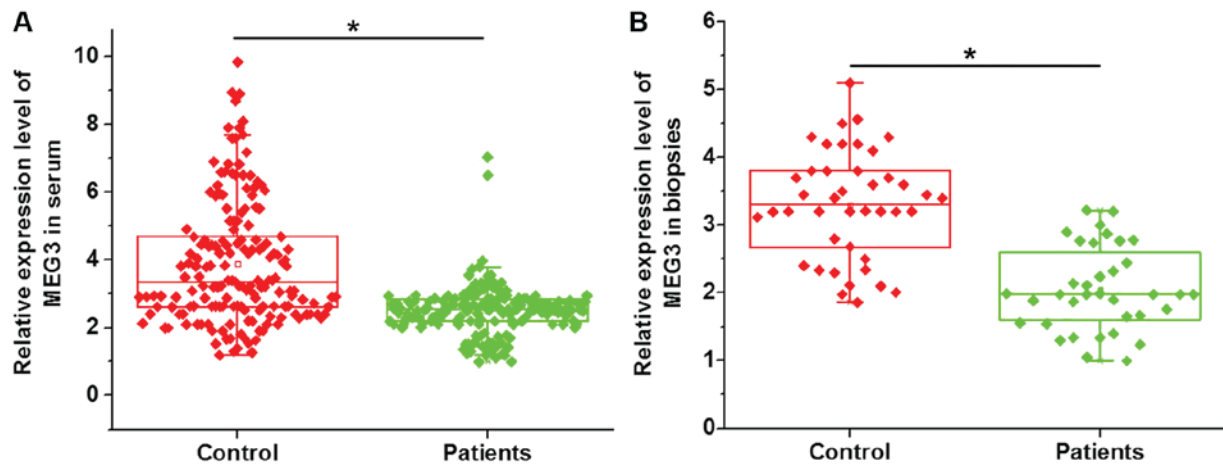


Figure 1. Comparison of expression levels of lncRNA-MEG3 in serum and open sacroiliac joint biopsies between ankylosing spondylitis patients and healthy controls. The normalized expression levels of lncRNA-MEG3 in (A) serum and (B) open sacroiliac joint biopsies of ankylosing spondylitis patients (n=172) and healthy controls (n=98) are provided. *P<0.05. Data are expressed as the minimum value, lower quartile, median, upper quartile and the maximum value. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3.

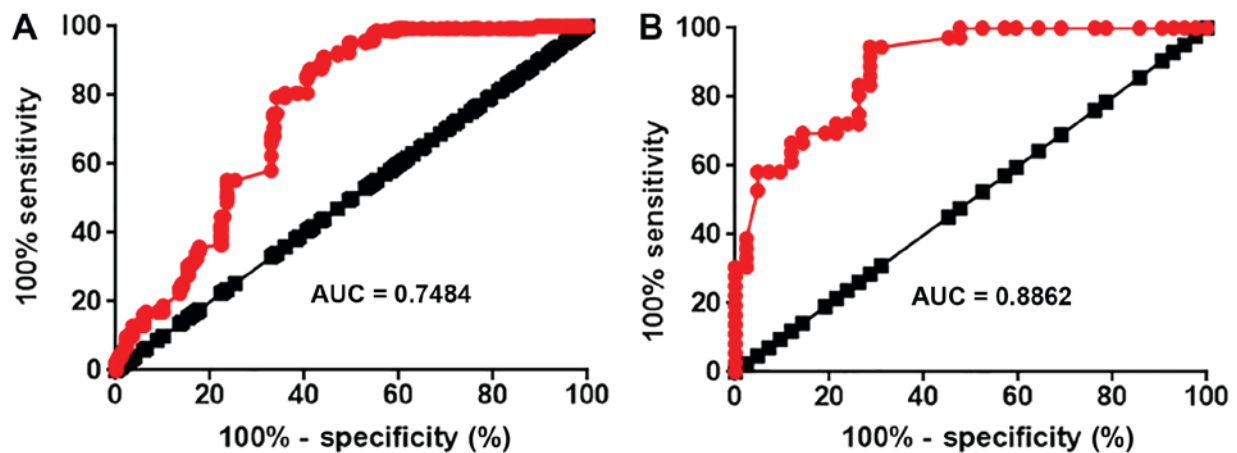


Figure 2. Diagnostic value of lncRNA-MEG3 expression in serum and open sacroiliac joint biopsies. The receiver operating characteristic curves for the use of lncRNA-MEG3 expression in (A) serum and in (B) open sacroiliac joint biopsies for the diagnosis of ankylosing spondylitis are provided. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3.

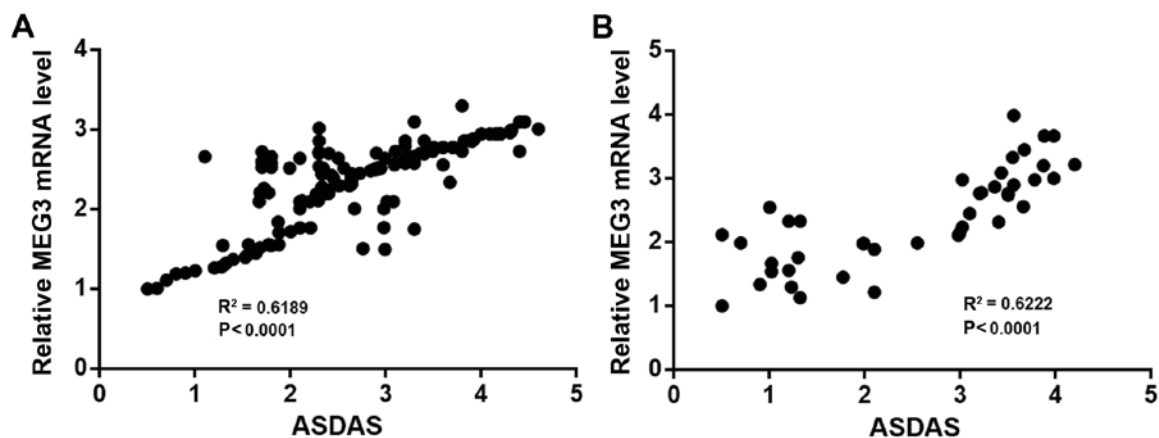


Figure 3. MEG3 mRNA expression in the serum and open sacroiliac joint biopsies are positively correlated with ASDAS. mRNA expression levels of MEG3 in (A) the serum and (B) open sacroiliac joint biopsies. ASDAS, ankylosing spondylitis disease activity score; MEG3, maternally expressed gene 3.

respectively, the serum circulating MEG3 levels and the expression levels of MEG3 in open sacroiliac joint biopsies

were not significantly correlated with the patients' sex, age and lifestyle habits, including smoking and drinking. However,

Table I. Association of circulating MEG3 levels with clinicopathological data of ankylosing spondylitis patients.

Variables	Cases (n)	High-expression group [n (%)]	Low-expression group [n (%)]	χ^2	P-value
Sex				0.095	0.76
Male	98	48 (49.0)	50 (51.0)		
Female	74	38 (51.4)	36 (48.6)		
Age (years)				0.374	0.54
≥ 25	80	38 (47.5)	42 (52.5)		
< 25	92	48 (52.2)	44 (47.8)		
Disease duration (years)				18.241	< 0.0001
≥ 5	84	28 (33.3)	56 (66.7)		
< 5	88	58 (65.9)	30 (34.1)		
ASDAS				19.066	< 0.001
< 1.3	32	17 (53.1)	15 (46.9)		
1.3-2.1	46	22 (47.8)	24 (52.2)		
2.1-3.5	44	24 (54.5)	20 (45.5)		
> 3.5	50	24 (48.0)	26 (52.0)		
Smoking				0.212	0.65
Yes	77	37 (48.1)	40 (51.9)		
No	95	49 (51.6)	46 (48.4)		
Drinking				1.147	0.28
Yes	93	43 (46.2)	50 (53.8)		
No	79	43 (54.4)	36 (45.6)		

ASDAS, ankylosing spondylitis disease activity score; MEG3, maternally expressed gene 3.

significant differences in the disease duration and ASDAS were identified between the high- and low-level MEG3 expression groups.

Effects of serum levels of circulating MEG3 on hospitalization time and re-hospitalization rate. According to the median expression levels of MEG3, patients were divided into high-level and low-level groups (n=86), and the hospitalization time and re-hospitalization rate were compared between the two groups. As presented in Fig. 4A, the hospitalization time in the low-level group was significantly longer than that in the high-level group ($P < 0.05$). In addition, the re-hospitalization rate in the low-expression group (30 out of 86, 34.9%) within 2 years after discharge was also higher than that in the high-level group (19 out of 86, 22.1%; Fig. 4B). However, the difference in the re-hospitalization rate between the two groups was not significant ($P = 0.06$).

Discussion

The onset, development and progression of ankylosing spondylitis are complex processes with multiple internal and external factors involved (1). It has been reported that HLA-B27 subtypes and bacteria are likely to be involved in the pathogenesis of ankylosing spondylitis (4). Furthermore, the development of ankylosing spondylitis is usually accompanied with changes in expression patterns of a large set of genes, including miRNAs and lncRNAs, indicating the involvement of lncRNAs in this

disease (14,15). However, studies on the detailed expression patterns of those lncRNAs, as well as their prognostic and diagnostic values for this disease, remain insufficient. In a recent study, Li *et al* (16) characterized the expression pattern of lncRNA-AK001085 in the blood of patients with ankylosing spondylitis and revealed that lncRNA-AK001085 was frequently downregulated in ankylosing spondylitis patients compared with that in healthy controls. lncRNA MEG3 has been proved to be overexpressed in patients with osteoporosis (9), which has similar mechanisms to those of ankylosing spondylitis (10). In the present study, a significantly downregulated MEG3 expression in the serum and open sacroiliac joint biopsies of ankylosing spondylitis patients compared with that in healthy controls was identified, indicating that downregulation of MEG3 is likely to be involved in ankylosing spondylitis. The opposite expression pattern of MEG3 in ankylosing spondylitis and osteoporosis may be explained by the inverse pathological processes between those two diseases (17). In the present study, marked overlaps in the levels of MEG3 between patients and healthy controls were observed, which indicates that differences between individuals may prevail.

The development of diseases is always accompanied with changes of certain substances (e.g. circulating lncRNAs) in the blood (18). Therefore, the detection of the changes of those substances may provide references for the diagnosis of specific diseases (18). In the present study, ROC curve analysis indicated that serum circulating MEG3 may be used to effectively distinguish patients with ankylosing spondylitis

Table II. Correlation between expression levels of MEG3 in open sacroiliac joint biopsies with clinicopathological data of ankylosing spondylitis patients.

Variables	Cases (n)	High-expression group [n (%)]	Low-expression group [n (%)]	χ^2	P-value
Sex				1.71	0.79
Male	28	12 (42.9)	16 (57.1)		
Female	14	9 (64.3)	5 (35.7)		
Age (years)				0.47	0.49
≥ 25	30	14 (46.7)	16 (53.3)		
< 25	12	7 (58.3)	5 (41.7)		
Disease duration (years)				6.11	0.01
≥ 5	20	6 (30.0)	14 (70.0)		
< 5	22	15 (68.2)	7 (31.8)		
ASDAS				6.27	0.01
< 1.3	10	7 (70.0)	3 (30.0)		
1.3-2.1	8	6 (75.0)	2 (25.0)		
2.1-3.5	12	4 (33.3)	8 (66.7)		
> 3.5	12	4 (33.3)	8 (66.7)		
Smoking				0.87	0.35
Yes	23	10 (43.5)	13 (56.5)		
No	19	11 (57.9)	8 (42.1)		
Drinking				1.56	0.21
Yes	24	10 (41.7)	14 (58.3)		
No	18	11 (61.1)	7 (38.9)		

ASDAS, ankylosing spondylitis disease activity score; MEG3, maternally expressed gene 3.

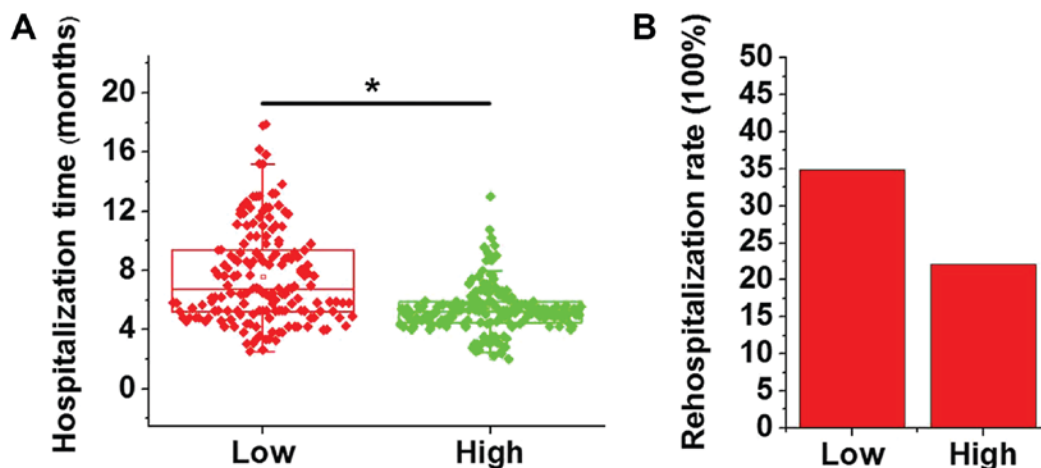


Figure 4. Effects of serum levels of circulating MEG3 on hospitalization time and re-hospitalization rate within 2 years after discharge of patients with high (n=86) and low (n=86) serum levels of MEG3 is provided. Hospitalization times are expressed as the minimum value, lower quartile, median, upper quartile and the maximum value. *P<0.05. MEG3, maternally expressed gene 3.

from healthy individuals. It is known that the expression of certain lncRNAs may be affected by tobacco consumption and alcohol abuse (19). In addition, a genome-wide alteration of lncRNA expression was identified with aging (20). In the present study, serum circulating MEG3 levels were not significantly associated with age, sex or the patients' smoking and drinking habits, but were significantly associated with the

disease duration and disease activity. These results suggest that serum circulating MEG3 may serve as a reliable and effective diagnostic biomarker for ankylosing spondylitis. Our future studies will work on the optimization of specificity, sensitivity and other parameters. Although various serum lncRNAs have a promising diagnostic value for multiple human diseases (21), the serum levels of circulating lncRNAs

may fail to fully reflect the expression pattern in lesion tissues. In the present study, it was observed that 3 patients with serum levels of MEG3 above the median value had expression levels of MEG3 in open sacroiliac joint biopsies below the median values. In another 6 patients, the serum levels of MEG3 below the median and expression levels of MEG3 in open sacroiliac joint biopsies above the median were observed. In addition, the ROC curve analysis indicated that the expression levels of MEG3 in open sacroiliac joint biopsies have a higher diagnostic value for ankylosing spondylitis compared with that of the serum circulating levels of MEG3. However, open sacroiliac joint biopsy as an invasive technique is unbearable for all patients (only 42 out of 172 patients were willing to receive it), particularly teenagers. Furthermore, the patients' health condition and availability of financial resources should be fully considered when selecting the diagnostic techniques. In the present study, the serum levels of circulating MEG3 were also identified to be significantly correlated with the ASDAS scores, which reflect the activity of ankylosing spondylitis, indicating the possible role of MEG3 as a marker for determining disease activity. In addition, a lower expression level of MEG3 was identified to be associated with a longer hospitalization time and a higher re-hospitalization rate within 2 years after discharge. These results suggest that serum circulating MEG3 may be used to guide the treatment and prognosis of ankylosing spondylitis.

The present study did not identify the downstream targets of MEG3 in ankylosing spondylitis. It has been reported that angiogenesis (22) and inflammation (23) have pivotal roles in the pathogenesis of ankylosing spondylitis. A previous study indicated that MEG3 knockdown aggravates retinal vessel dysfunction by promoting inflammatory responses (24). In addition, an inverse association between MEG3 and VEGF has been observed in osteoarthritis (25). Therefore, a reduction in MEG3 may participate in ankylosing spondylitis through inducing inflammation and angiogenesis. Besides the ASDAS, other important indicators, including the erythrocyte sedimentation rate, C-reactive protein and procalcitonin should also be analyzed. In the present study those indicators were not detected due to the limited resources, but this will be addressed in future studies.

In conclusion, the present study revealed that MEG3 is downregulated in ankylosing spondylitis patients compared with that in healthy controls. Serum circulating MEG3 and MEG3 expression in sacroiliac joint biopsies are biomarkers for ankylosing spondylitis. The expression levels of MEG3 were not associated with the patients' age, sex and smoking/drinking habits, but were associated with the duration of disease and closely correlated with the disease activity. Furthermore, patients with lower expression levels of MEG3 had a longer hospitalization time and a higher re-hospitalization rate within 2 years after discharge. It may therefore be concluded that lncRNA MEG3 is downregulated in ankylosing spondylitis and it may hold predictive value regarding disease activity, disease duration, hospitalization time and re-hospitalization rate within 2 years after discharge.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL and ZL designed experiments. WL, LH and CZ performed experiments. WL, LH and ZL analyzed data. ZL interpreted data and drafted the manuscript. All authors read and approved the manuscript.

Ethical approval and consent to participate

The present study was approved by the ethics committee of Yongchuan Hospital of Chongqing Medical University (Chongqing, China) and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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