

H19 Increases IL-17A/IL-23 Releases via Regulating VDR by Interacting with miR675-5p/miR22-5p in Ankylosing Spondylitis

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Long non-coding RNA (lncRNA) H19 is associated with inflammatory diseases, but the molecular mechanism of H19 in the inflammatory process of ankylosing spondylitis (AS) is unclear. Here, we investigated the role of H19 and its downstream molecules in the inflammation of AS by microarray analysis, qRT-PCR, western blot, and dual-luciferase reporter assay. H19 small interfering RNA (siRNA) (Si-H19) and adenovirus (AD-H19) were used to decrease and increase H19 expression, respectively. 42 annotated lncRNAs were identified, and H19 was overexpressed. H19, vitamin D receptor (VDR), and transforming growth factor β (TGF- β) can bind to microRNA22-5p (miR22-5p) and miR675-5p. Si-H19 significantly downregulated miR22-5p and upregulated miR675-5p expression; Si-H19 decreased the protein and mRNA expression of VDR and decreased the cytokine and mRNA levels of interleukin-17A (IL-17A) and IL-23. These results were verified by AD-H19. In addition, miR22-5p and miR675-5p inhibitors increased the protein and mRNA expression of VDR and increased the cytokine and mRNA levels of IL-17A and IL-23. These results were also confirmed by miRNA mimics. Furthermore, H19 directly interfered with miR22-5p and miR675-5p expression, whereas the two miRNAs directly inhibited VDR expression. Overall, the H19-miR22-5p/miR675-5p-VDR-IL-17A/IL-23 signaling pathways have important roles in the pathogenesis of AS.

INTRODUCTION

Ankylosing spondylitis (AS) is a form of inflammatory arthritis of the spine and sacroiliac joint that is highly heritable and familial. Large-scale studies in different populations have identified many genetic polymorphisms and inflammatory pathways related to the etiology and pathogenesis of AS,¹⁻⁶ but the detailed mechanisms for the underlying inflammatory process of AS remain unclear. Non-coding RNAs (ncRNAs) account for more than 80% of human genomic transcripts and involved in many biological processes including transcriptional and post-transcriptional regulation, mRNA stability and

translation, and protein degradation and transport.⁷ However, few studies have investigated the role of ncRNAs in AS.

MicroRNA (miRNA) is a small ncRNA containing about 20 nucleotides that downregulates the expression of target genes by mediating mRNA decay and inhibiting translation.⁸ Increasing studies have shown that miRNAs play an important role in cellular function and inflammation. Abnormal miRNA expression has been observed in peripheral blood mononuclear cells (PBMCs) and T cells in AS patients.⁹⁻¹¹ Nonetheless, little is known about the upstream regulator of miRNA, which may have the potential to block the inflammatory pathway of AS. Long ncRNA (lncRNA) is a class of single-stranded ncRNAs with more than 200 nucleotides. It is transcribed and processed like mRNA but it cannot encode functional proteins. Hitherto, the functions of many lncRNAs are rarely known.¹²

Recently, lncRNAs have been considered as competing endogenous RNAs (ceRNAs) that sponge miRNAs' complementary base pairing.¹³⁻¹⁵ lncRNA H19 activates osteoblast differentiation via activating Wnt/ β -catenin signal pathway by acting as a ceRNA for miR22 and miR141.¹⁵ In addition, H19 can act as an upstream molecule of miRNA to regulate mRNA expression, and miR675-5p is transcribed from the first exon of H19 and has been validated to play an important role in some diseases.^{16,17} These findings strongly support the regulatory role of H19 in the development of inflammatory diseases.¹⁸ Vitamin D receptor (VDR) is a nuclear receptor that mediates the production of 1,25(OH)₂D₃, and transforming growth factor β (TGF- β) is a multi-functional cytokine involved in regulation and cell proliferation.

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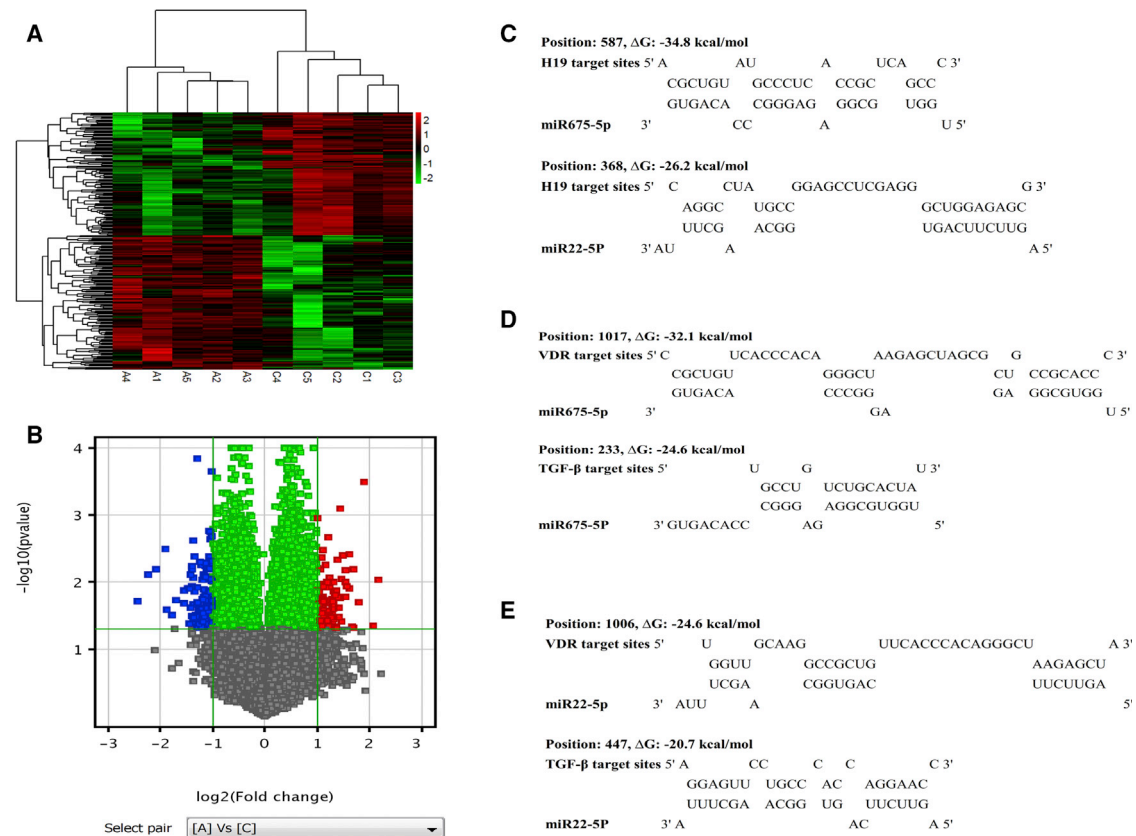


Figure 1. Differentially Expressed lncRNAs, and Bioinformatics Analysis

(A) Hierarchical cluster analysis showed distinguishable expression of lncRNAs between patients and controls. Each row represents one lncRNA (A1–A5 and C1–C5 represent an individual code of patients and controls, respectively), and each column corresponds to the expression profile of one sample. Red pixels indicate higher expression of genes, whereas green pixels indicate lower expression. (B) Volcano plot of lncRNAs. The abscissa is \log_2 (2.0-FC in expression), and the ordinate is $-\log_{10}$ (p value). The red and blue dots correspond to genes that are upregulated and downregulated, respectively, the green dots correspond to genes with an absolute value < 2 in FC, and the black dots correspond to those genes that are not significantly different (A and C represent the individual code of patients and controls, respectively). (C–E) Schematic diagrams of mutual interplays between H19 and miR675-5p/miR22-5p (C), miR675-5p and VDR/TGF- β (D), and miR22-5p and VDR/TGF- β (E). The positions of miRNA binding sites and the calculated ΔG values are shown (kcal/mol).

Previous studies have shown that VDR and TGF- β have important roles in inflammation and AS,^{17,19,20} and their relationship with H19 and miRNA has been well investigated in the development of inflammatory diseases.^{17,21} In patients with ulcerative colitis, H19 overexpression may decrease the expression of VDR, and H19 promotes osteoblast differentiation and enhances heterotopic bone formation via activating TGF- β /Smad3/HDAC pathway by deriving miR675.²¹

A number of differentially expressed (DE) lncRNAs have been identified in AS patients.^{22–29} However, it is unclear how lncRNA and its downstream molecules affect the development of AS. In addition, an online-based program RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/>) found that miR22-5p and miR675-5p binding sites of H19, VDR, and TGF- β ; IL-17A, IL-23, and tumor necrosis factor alpha (TNF- α) play important roles in the pathogenesis of AS.^{30–33} In this study, we investigated the role of H19 and its downstream signaling molecules in the inflammatory process of AS.

RESULTS

lncRNA Expression Profiles of Peripheral Blood Mononuclear Cell of AS

To identify DE lncRNAs in peripheral blood mononuclear cells (PBMCs) of AS, we carried out lncRNA expression profiles in 5 AS patients and 5 healthy controls (HCs) using lncRNA microarray. Heatmap and volcano plots showed that lncRNAs distinguished AS patients from HCs (Figures 1A and 1B). A total of 154 lncRNAs were DE in PBMCs (fold change [FC] > 2 , $p < 0.05$), including 71 lncRNAs that were upregulated, 83 that were downregulated, and 42 that were annotated (Table S1).

Bioinformatics Analysis

To explore whether VDR and TGF- β could be regulated by miRNA, we first analyzed the 3' UTR of VDR and TGF- β using the RNAhybrid online program³⁴ and found several potential miRNA-binding sites. Similarly, we used the program to predict

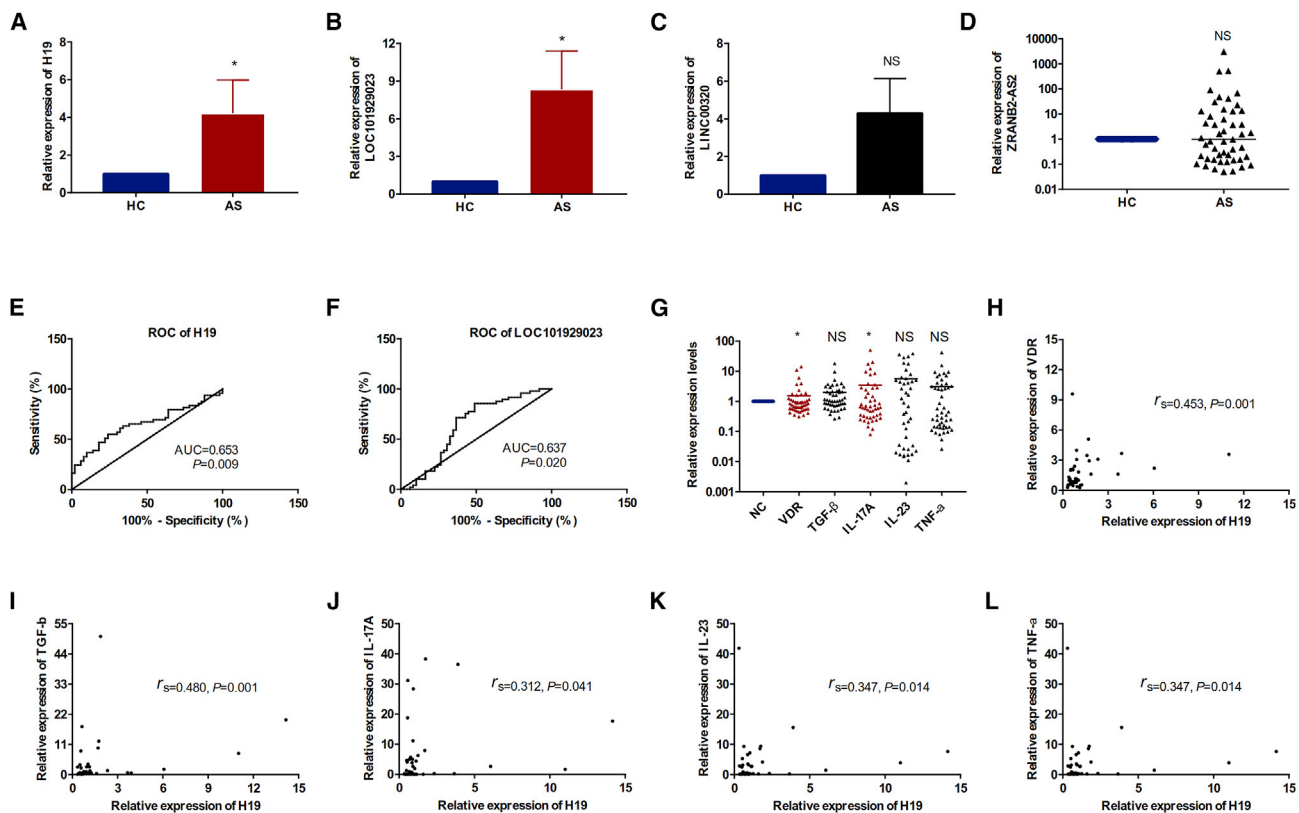


Figure 2. Determination of Candidate lncRNA and Its Correlation with mRNAs

(A–D) Relative expression of H19 (A), LOC101929023 (B), LINC00320 (C), and ZRANB2-AS2 (D) between AS and HC. (E and F) ROC results of H19 (E) and LOC101929023 (F). (G) Expression of VDR, TGF- β , IL-17A, IL-23, and TNF- α . (H–L) Correlation analysis between H19 and VDR (H), TGF- β (I), IL-17A (J), IL-23 (K), and TNF- α (L). * $p < 0.05$; NS, not significant as compared with the HC group. AS, ankylosing spondylitis; AUC, area under curve; HC, healthy control; ROC, receiver operating characteristic.

the binding sites H19 and miRNAs. According to bioinformatics results and previous studies,^{15,17,21} miR22-5p and miR675-5p were selected as the target molecules for H19 (Figure 1C) and VDR and TGF- β as the targets for miR22-5p and miR675-5p (Figures 1D and 1E).

Validation of DE lncRNAs in PBMCs

Subsequently, four lncRNAs were selected to verify the microarray results using qRT-PCR in 49 AS patients and 49 HCs. lncRNA H19 and LOC101929023 (Figures 2A and 2B) but not LINC00320 or ZRANB2-AS2 (Figures 2C and 2D) were significantly overexpressed in AS patients compared to HCs, suggesting that H19 and LOC101929023 are really DE lncRNAs in AS. To further determine the most representative lncRNA, we performed receiver operating characteristic (ROC) curve analyses on the two dysregulated lncRNAs and found that H19 had a higher area under curve (AUC) and a greater power (p value) than LOC101929023 (Figures 2E and 2F), suggesting that the diagnostic value of H19 is greater than LOC101929023. Therefore, H19 was selected as the target lncRNA for the next investigations.

mRNA Expression in PBMC

In addition to validating the microarray results, we also measured the mRNA expression of VDR, TGF- β , IL-17A, IL-23, and TNF- α in the same cohort of 49 patients and 49 HCs. We found that the expression of VDR and IL-17A but not the other three mRNAs was significantly higher in AS patients compared to HCs (Figure 2G), indicating that VDR and IL-17A are important for the inflammation of AS. Furthermore, we carried out correlation analyses to explore the potential relationship between H19 and the five mRNAs. We found that the expression of H19 was positively associated with the mRNAs, and these associations provide clues to further explore the regulatory function of H19, although they are weak (Figures 2H–2L).

H19 Regulates the Target Molecules in PBMCs

To explore the role of H19 in the inflammatory responses, we changed the expression of H19 (i.e., H19 knockdown and overexpression) in PBMC. First, we designed three specific siRNA sequences (coded as Si-H19-1484, Si-H19-814, and Si-H19-595) to inhibit H19 expression and used a negative control (NC) as the internal control group. Compared to the NC group, both Si-H19-1484 and Si-H19-814 but not Si-H19-595 significantly downregulated H19 expression, and

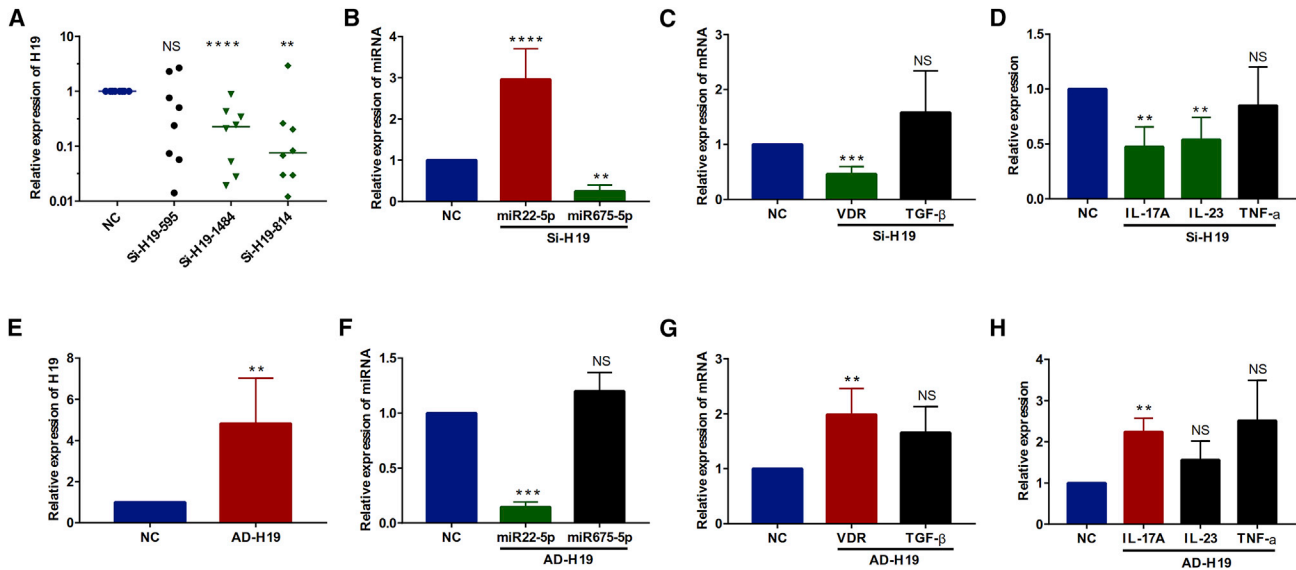


Figure 3. H19 Regulates Target Molecules

(A) Si-H19-1484 and Si-H19-814 but not Si-H19-595 significantly decreased the expression of H19. (B–D) Si-H19 significantly increased the expression of miR22-5p and decreased the expression of miR675-5p (B), VDR (C), IL-17A (D), and IL-23 (D). (E–H) AD-H19 significantly increased the expression of H19 (E), decreased the expression of miR22-5p (F), and increased the expression of VDR (G) and IL-17A (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; NS, not significant as compared with the NC group. AD-H19, H19 overexpression; Si-H19, H19 knockdown; NC, negative control.

the knockdown effect of Si-H19-1484 was stronger (Figure 3A). Therefore, we used Si-H19-1484 to inhibit H19 expression in subsequent experiments. We found that Si-H19 significantly decreased the expression of miR675-5p and VDR and increased the expression of miR22-5p (Figures 3B and 3C). In addition, Si-H19 significantly decreased the mRNA expression of IL-17A and IL-23 but not TNF- α (Figure 3D).

Second, to confirm the above findings, PBMCs were transfected with H19-associated adenovirus (AD-H19) to increase the expression of H19. Compared to the NC group, AD-H19 significantly increased the expression of H19 (Figure 3E), suggesting that AD-H19 is effective. In contrast to the Si-H19 results, AD-H19 increased the expression of VDR and IL-17 and decreased the expression of miR22-5p (Figures 3F–3H). These findings indicated that H19 has an important role in the development of AS by regulating miR22-5p, miR675-5p, and VDR, which needs further validation.

miRNAs Regulate Target Molecules and Reverse Regulate H19 in PBMCs

To further investigate the role of H19 and its downstream molecules, we transfected PBMCs with miRNA mimics (i.e., miR22-5p and miR675-5p mimics) and miRNA inhibitors (i.e., miR22-5p and miR675-5p inhibitors) to increase and decrease the expression of miRNAs, respectively. First, the expression of miR22-5p and miR675-5p was increased by miRNA mimics and decreased by miRNA inhibitors (Figures 4A and 4E), suggesting that these mimics and inhibitors are useful. Second, to explore the effects of the two

miRNAs on H19, we measured the expression of H19 when cells were transfected with miRNA mimics and inhibitors. The two miRNA inhibitors significantly increased H19 expression, and only miR22-5p mimics decreased H19 expression (Figures 4B and 4F), suggesting that miRNAs have a negative feedback role in H19. Third, miR22-5p mimics decreased the mRNA expression of VDR, TGF- β , IL-17A, and IL-23, whereas miR22-5p inhibitors increased their expression levels (Figures 4C and 4D); miR675-5p mimics and inhibitors had similar findings in all molecules except for TGF- β mRNA expression (Figures 4G and 4H). Furthermore, the expression of TNF- α was not changed by the two miRNA mimics and inhibitors, indicating that TNF- α may not be a key molecule for AS (Figures 4D and 4H). These results suggested that the H19-miRNA (miR22-5p and miR675-5p)-VDR-IL-17A/IL-23 pathway may play a key role in AS.

H19 and miRNAs Regulate VDR and TGF- β Proteins in PBMCs

To explore the effects of H19, miR22-5p, and miR675-5p on post-transcriptional regulation, we measured the protein expression of VDR and TGF- β using western blot. As expected, the results of proteins were consistent with those of mRNAs. The protein expression of VDR and TGF- β was significantly decreased by Si-H19 and increased by AD-H19 (Figures 5A–5C). Moreover, both miR22-5p and miR675-5p mimics significantly inhibited the protein expression of VDR and TGF- β , and miR22-5p inhibitors increased their expression levels (Figures 5D–5I). However, the miR675-5p inhibitors increased the protein expression of VDR but not TGF- β (Figure 5I). Therefore, H19, miR22-5p, and miR675-5p have important roles in VDR and

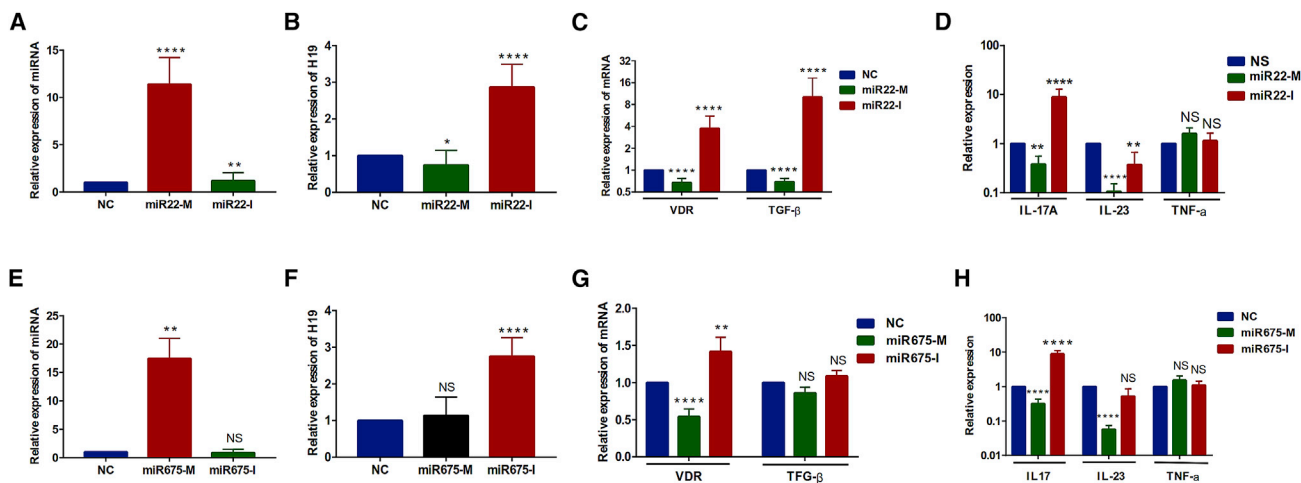


Figure 4. miRNAs Regulate Target Molecules and Reverse the Regulation of H19

(A) Effect of miR22-5p mimics and inhibitors on miR22-5p expression. (B–D) The miR22-5p mimics significantly decreased the expression of H19 (B), VDR (C), TGF- β (C), IL-17A (D), and IL-23 (D), while the miR22-5p inhibitors increased their expressions. (E) Effect of miR675-5p mimics and inhibitors on miR675-5p expression. (F–H) The miR675-5p mimics significantly decreased the expression of VDR (G), TGF- β (G), IL-17A (H), and IL-23 (H), while miR675-5p inhibitors increased the expressions of H19 (E), VDR (G), IL-17A (H), and IL-23 (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; NS, not significant as compared with the NC group. miR22-M, miR22-5p mimics; miR22-I, miR22-5p inhibitors; miR675-M, miR675-5p mimics; miR675-I, miR675-5p inhibitors; NC, negative control.

TGF- β proteins, especially VDR protein. Given the above results, two signaling pathways (H19-miR675-5p-VDR and H19-miR22-5p-VDR) may be involved in the inflammatory mechanisms of AS.

H19 Directly Regulates miRNAs, and miRNAs Directly Target VDR

miRNA directly suppresses the expression of target genes⁸ while lncRNA acts as a posttranscriptional regulator by interacting with miRNA via ceRNA or miRNA sponge.^{13,14} To further investigate whether H19 is a ceRNA or molecular sponge in the regulation of miR22-5p and miR675-5p, and to investigate the role of the two miRNAs in the regulation of VDR, we performed luciferase reporter gene assays in HEK293 T cells. Luciferase reporter gene vectors (i.e., mutant-type named H19-MUT, VDR-MUT containing a mutation at the predicted miR22-5p and miR675-5p and wild-type named H19-WT, VDR-WT) at the predicted miR22-5p and miR675-5p (Figures 1C–1E) binding sites were constructed, and psiCHECK2 empty vector is a NC; these vectors were co-transfected into cells with miR22-5p mimics or miR675-5p mimics.

The luciferase activity of H19-WT and VDR-WT was suppressed by miR22-5p mimics (Figures 6A and 6B) and miR675-5p mimics (Figures 6C and 6D); and miR675-5p mimics showed a trend toward decreasing the luciferase activity of H19-MUT, indicating that the relationship between H19 and miR675-5p is diverse.^{15,21} However, the luciferase activity of H19-MUT and VDR-MUT in cells transfected with the two miRNA mimics showed no statistical differences (Figures 6A–6D). These findings suggested that H19 and the 3' UTR of VDR can bind to miR22-5p and miR675-5p and that H19 may serve as a ceRNA for miR22-5p, interact directly with miR675-5p,

and further compete with VDR for miR22-5p and miR675-5p binding.

H19 and miRNAs Regulate Inflammatory Cytokines in PBMCs

To investigate the effects of H19, miR22-5p, and miR675-5p on key cytokines in the inflammatory pathway of AS and to further validate the expression of mRNAs, we measured the level of IL-17A, IL-23, and TNF- α cytokines in cell supernatants. Si-H19 significantly decreased the levels of IL-17A and IL-23 cytokines, and AD-H19 significantly increased their levels (Figures 7A and 7B). However, neither Si-H19 nor AD-H19 altered TNF- α level (Figure 7C). Although both miRNA inhibitors significantly increased the IL-17A level, only miR22-5p inhibitors increased the IL-23 level (Figures 7D and 7E). In addition, the two miRNA mimics did not change the level of IL-17A and IL-23 cytokines, but miR22-5p mimics reduced TNF- α level (Figures 7D–7F). Taken together, H19, miR22-5p, and miR675-5p play important roles in the release of inflammatory cytokines, especially IL-17A and IL-23.

DISCUSSION

In this study, we identified 42 annotated DE lncRNAs in AS patients where lncRNA H19 was significantly overexpressed. Moreover, H19 increased the release of IL-17A and IL-23 cytokines by competitively binding to VDR via acting as a ceRNA for miR22-5p and interacting with miR675-5p. These findings provide new insights into the role of H19 and its downstream molecules in the pathogenesis and inflammatory process of AS.

AS is an inflammatory disease without definitive mechanisms, and previous microarray and sequencing studies have shown that there

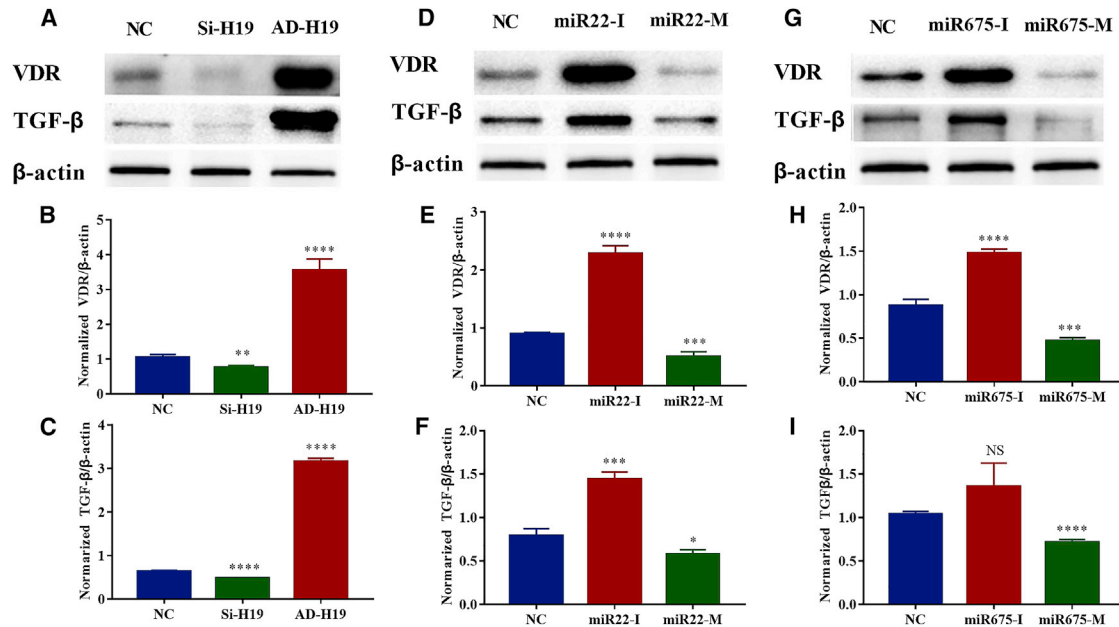


Figure 5. H19, miR675-5p, and miR22-5p Regulate Protein Expression

(A–C) Effect of H19 on the protein expression of VDR and TGF- β . Si-H19 significantly decreased the protein expression of VDR (B) and TGF- β (C), while AD-H19 significantly increased their expressions. (D–F) Effect of miR22-5p on the protein expression of VDR (E) and TGF- β (F); miR22-5p inhibitors significantly increased the protein expression of VDR (E) and TGF- β (F) while miR22-5p mimics decreased their expressions. (G–I) Effect of miR675-5p on the protein expression of VDR (H) and TGF- β (I), miR675-5p inhibitor increased the protein expression of VDR (H), while miR675-5p mimics decreased the protein expression of VDR (H) and TGF- β (I). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; NS, not significant as compared with the NC group. AD-H19, H19 overexpression; Si-H19, H19 knockdown; miR22-M, miR22-5p mimics; miR22-I, miR22-5p inhibitors; miR675-M, miR675-5p mimics; miR675-I, miR675-5p inhibitors; NC, negative control.

are several DE lncRNAs in AS,^{26,27} suggesting that the involvement of lncRNAs in AS, but functionalities of lncRNAs, have not been characterized. In this study, many dysregulated lncRNAs were found in PBMCs of AS, and the expressions of lncRNAs were detected by microarray analysis and verified by qRT-PCR, which are routinely used for identifying RNA.^{27,35} The two testing methods showed similar trends, but FCs were not exactly the same. One possible explanation is the different sensitivity of the two assays or the use of different samples in the two phases.

Our results indicated that H19 and LOC101929023 were substantially overexpressed in AS patients and the AUC of H19 was higher than LOC101929023. Therefore, H19 was selected as a candidate lncRNA in this study. H19 is highly expressed from the early stages of embryogenesis throughout fetal life in many tissues and is downregulated postnatally;³⁶ recent studies found that the H19 regulates cell differentiation and musculoskeletal system regeneration^{21,37–40} and plays an important role in inflammatory diseases.^{17,35,41–43} For example, H19 expression was higher in synovial tissue from patients with rheumatoid arthritis and osteoarthritis compared to controls,⁴¹ a microarray analysis revealed 121 DE lncRNAs in osteoarthritis patients and H19 was upregulated.³⁵ These findings suggest that H19 is a key molecular in the pathogenesis of inflammatory diseases, and we expect more studies will be conducted to explore the role of H19 in other diseases.

The bioinformatic analysis found that H19 and the 3' UTR of VDR and TGF- β can bind to the miR22-5p and miR675-5p, previous studies have also reported,^{15,17,21,44} and one single miRNA can target several mRNAs and one mRNA can also be regulated by many miRNAs.^{45,46} Furthermore, H19 was enriched in AGO2 (a major constituent of the RNA-induced silencing complex), suggesting that H19 is a natural decoy of miRNA.⁴⁷ More importantly, two different potential modulatory mechanisms of H19 involved in cell differentiation and inflammatory progression have been investigated⁴⁷ (see Figure 8). First, one is that H19 serves as a ceRNA by sponging for miRNAs, thereby interfering with gene regulatory networks.^{15,44} Our findings of the overexpression of H19 decreased miR22-5p expression and increased the mRNA expression of VDR, and these results were confirmed by Si-H19. The protein expression of VDR and TGF- β was enhanced by AD-H19 and suppressed by Si-H19; these findings were similar to their mRNAs results, suggesting that H19 can regulate gene expression at the transcriptional and post-transcriptional levels. To further validate the modulatory mechanisms of H19, we examined whether miR22-5p could inhibit the expression of H19 and downstream target genes. Our results showed that the expression of H19 was suppressed by miR22-5p mimics and amplified by miR22-5p inhibitors, further suggesting that miR22-5p can reverse the effect of H19^{15,44} and lncRNA can regulate miRNA and vice versa. Previous studies found that H19 activated Wnt/ β -catenin signaling by acting as a ceRNA for miR22.^{15,44} These studies strongly

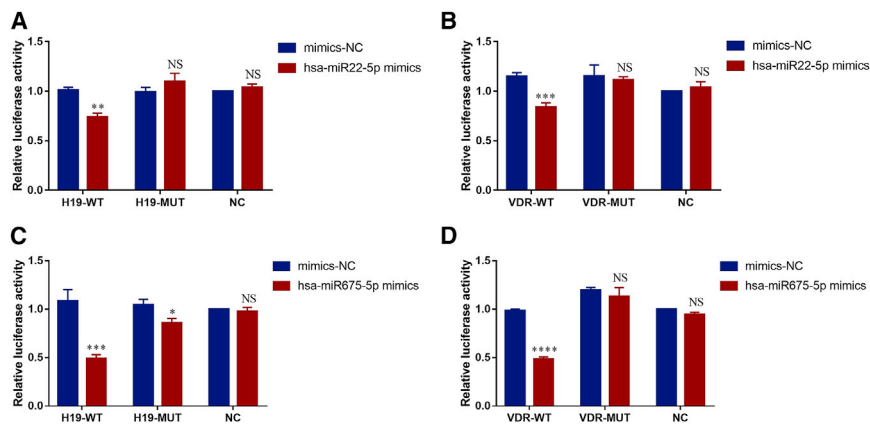


Figure 6. miR-22-5p and miR675-5p Interact with H19 and VDR via Direct Binding

(A–D) Wild-type or mutant-type H19 and VDR 3' UTR luciferase reporter gene vectors were constructed (named H19-WT, VDR-WT, H19-MUT, and VDR-MUT); mutant-type vectors contained a mutation at the predicted miR22-5p (A and B) and miR675-5p binding sites (C and D). The above vectors were co-transfected into HEK293T cells with miR22-5p mimics and inhibitors, or miR675-5p mimics and inhibitors; the luciferase activity was determined. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; NS, not significant as compared with the NC group. H19-WT, WT H19; H19-MUT, mutant-type H19; VDR-WT, wild-type VDR; VDR-MUT, mutant-type VDR; NC, negative control containing psiCHECK2 empty vector.

support the regulating effect of H19 on the disease process and cell function. Moreover, we found that miR22-5p mimics suppressed the mRNA level and protein expression of VDR, indicating that miR22-5p may be a useful regulator of VDR. In addition, the luciferase activity of H19 and VDR was suppressed by miR22-5p mimics, indicating that H19 directly regulates miR22-5p and miR22-5p regulates VDR. Taken together, H19 regulates the expression of VDR by acting as a ceRNA of miR22-5p. Second, another regulatory pathway of H19 is the network of lncRNA-miRNA-mRNA, which means that H19 modulates mRNA expression by interacting directly with miRNA. Unlike the relationship between H19 and miR22-5p, we found that H19 was positively associated with miR675-5p, and Si-H19 significantly decreased the expression of miR675-5p. We did not detect the effects of H19 and miR675-5p on the mRNA expression of TGF- β . However, H19 significantly altered the protein expression of TGF- β , and miR675-5p mimics slightly decreased the protein expression of TGF- β , indicating that miR675-5p regulation of the protein expression of TGF- β may be partially mediated by H19. Moreover, certain lncRNAs encode miRNAs and work together to achieve regulatory functions. miR675-5p is encoded by the first exon of H19 and acts as a cooperator of H19 in the regulation of cellular differentiation,^{15,48} but their relationship is mixed. H19 and miR675 were significantly upregulated in osteogenic differentiation and inhibited the mRNA and protein expression of TGF- β .²¹ However, another study has shown that miR675-5p makes a negative regulatory feedback loop by target binding site of H19 in osteogenic differentiation.¹⁵ H19 was inhibited by ectopic transfection of miR675-5p, and the overexpression of miR675-5p suppressed the luciferase activity of H19.¹⁵ These findings suggest that the roles of miR675-5p are diverse and the regulatory relationship between H19 and miR675-5p in inflammation needs further confirmation. We first investigated the role of VDR and the H19-miR675-5p-VDR pathway in the inflammation and pathogenesis of AS. The overexpression of H19 and miR675-5p mimics increased the mRNA level and protein expression of VDR in PBMCs. While in ulcerative colitis tissues, H19 overexpression decreased VDR expression, and this effect was significantly attenuated by co-transfection with miR675 inhibitor.¹⁷ Moreover, the luciferase activity of H19 and VDR was suppressed by miR675-5p mimics. Taking into account the findings of

H19-miR22-5p-VDR, these results further indicate that H19 is very important for the regulation of gene transcription and translation, future studies should focus on the role of signal pathways in AS and other inflammatory diseases.

Differs from the experiments of Transwell and wound healing in tumor research, we measured the mRNA expressions and cytokine levels of IL-17A, IL-23, and TNF- α in AS patients. Of the three cytokines, IL-17A is the most important to the inflammation of AS. The mRNA expression and cytokine levels of IL-17A were significantly increased by AD-H19, miR22-5p, and miR675-5p inhibitors, and these results were further confirmed by Si-H19 and miRNAs mimics. IL-23 but not TNF- α had similar results, indicating that the IL-17A/IL-23 signaling has an important role in the inflammatory progression of AS. IL-17 and IL-23 levels were elevated in serum and PBMCs supernatants in AS patients, and IL-23 could stimulate IL-17 production, indicating that IL-17 is strongly associated with IL-23,⁴⁹ and bone changes in AS patients were the combined effects of IL-23 and IL-17A.⁵⁰ Intriguingly, TGF- β and VDR are thought to be important regulators of the inflammatory response process,^{51–53} and VDR and TGF- β signaling pathways also play vital roles in the downstream cytokines including TNF- α and IL families. Taken together, we hypothesized that the abnormal expression of H19 alters mRNA expression by changing miRNAs' status in two different ways to increase IL-17A and IL-23 release in PBMCs, which may then enter into the spine to cause or exacerbate the inflammatory response of AS (Figure 8).

Our research includes several strengths. First, we developed a DE lncRNAs profile in AS patients and investigated the role of H19 and its downstream pathways in the inflammatory responses of AS. Second, PBMCs were directly extracted from human peripheral blood rather than purchased from cell lines. Human inflammation and immune-associated cells should be considered to explore the effects of our findings on AS in the future. In addition, PBMCs are easy to collect and low cost, so the detection of H19 in PBMCs may be widely used to identify AS patients in larger sample sizes. Previous studies suggested that disease progression is often accompanied by changes in biomarkers, which can provide guidance for the treatment of disease.⁵⁴ However, some limitations should be considered. First, the

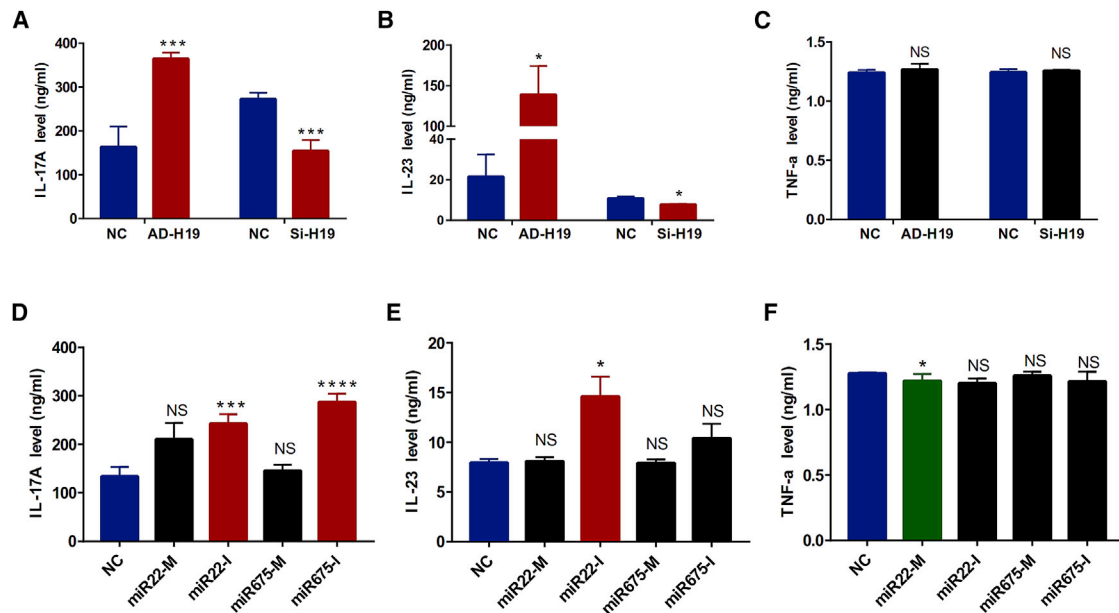


Figure 7. H19, miR675-5p, and miR22-5p Regulate Cytokine Levels

(A–C) AD-H19 significantly increased the level of IL-17A (A) and IL-23 (B) cytokines and Si-H19 decreased their levels, but the level of TNF- α cytokine (C) did not change when cells were transfected with adenovirus and siRNA. (D–F) miR22-5p and miR675-5p inhibitors significantly increased the level of IL-17A cytokines (D), and miR22-5p inhibitors increased the level of IL-23 cytokines (E), and miR22-5p mimics decreased the level of TNF- α cytokines (F). * $p < 0.05$, *** $p < 0.005$, **** $p < 0.001$; NS, not significant as compared with the NC group. AD-H19, H19 overexpression; Si-H19, H19 knockdown; miR22-M, miR22-5p mimics; miR22-I, miR22-5p inhibitors; miR675-M, miR675-5p mimics; miR675-I, miR675-5p inhibitors; NC, negative control.

underlying mechanisms of H19-associated pathways in different cell types contributing to AS pathogenesis need to be explored. Second, the role of TGF- β in the inflammatory response needs further validation. Finally, our results should be verified in animal models and other populations.

In conclusion, H19 is overexpressed in AS patients and mediates inflammatory progression by acting as a ceRNA in the axis of H19-miR22-5p-VDR-IL-17A/IL-23 and interaction with miRNA in the axis of H19-miR675-5p-VDR-IL-17A/IL-23. This study lays an important foundation for further understanding of the pathogenesis of AS and H19 may be a usefully diagnostic and therapeutic biomarker for AS.

MATERIALS AND METHODS

Subjects

Patients diagnosed with AS according to the 1984 modified New York criteria of the American College of Rheumatology were enrolled from the Department of Rheumatology and Immunology. Patients were excluded if they had been diagnosed with other inflammatory autoimmune diseases such as inflammatory bowel disease or rheumatic disease. Age- and sex-matched HCs without a history of autoimmune and chronic diseases were enrolled from the local community. Five pairs of AS patients and HCs were included for the screening of DE lncRNA using human lncRNA microarray, 49 pairs for lncRNA verification and mRNAs detection (i.e., VDR, TGF- β , IL-17A, IL-23, and TNF- α) using qRT-PCR, and 8 AS patients for functional

experiments. All participants provided written informed consent. This study was approved by the ethics committee of Anhui Medical University. Clinical characteristics of subjects are listed in Table S2.

PBMCs Extraction and Cell Culture

PBMCs were extracted from peripheral venous blood using a standard Ficoll-Hypaque density gradient centrifugation method according to the protocol. PBMCs were cultured in RPMI-1640 medium (Invitrogen-GIBCO, USA) using 12-well culture dishes containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. PBMCs were stimulated with phytohemagglutinin (PHA)^{55,56} at 37°C and 5% CO₂ to increase the number of cells. Cell viability was measured using a 0.4% trypan blue solution. Cells were harvested after incubation, and supernatants were collected and stored at –80°C for ELISA.

lncRNA Microarray Analysis

The protocol of Arraystar lncRNA array is as follows: (1) preparation, clearance, and quality control of RNA; (2) preparation of labeling reactions; (3) purification of labeled/amplified RNA and quality control of labeled cRNA; (4) hybridization; (5) microarray cleaning; (6) scanning; and (7) data extraction. The complementary DNA (cDNA) was labeled and hybridized using the 4 × 180 K human lncRNA microarray (Agilent, USA) designed for 89,459 lncRNA probes. DE genes with $p < 0.05$ and FC > 2 were selected. All microarray analyses were performed by the OE Biotech Company (Shanghai, China).

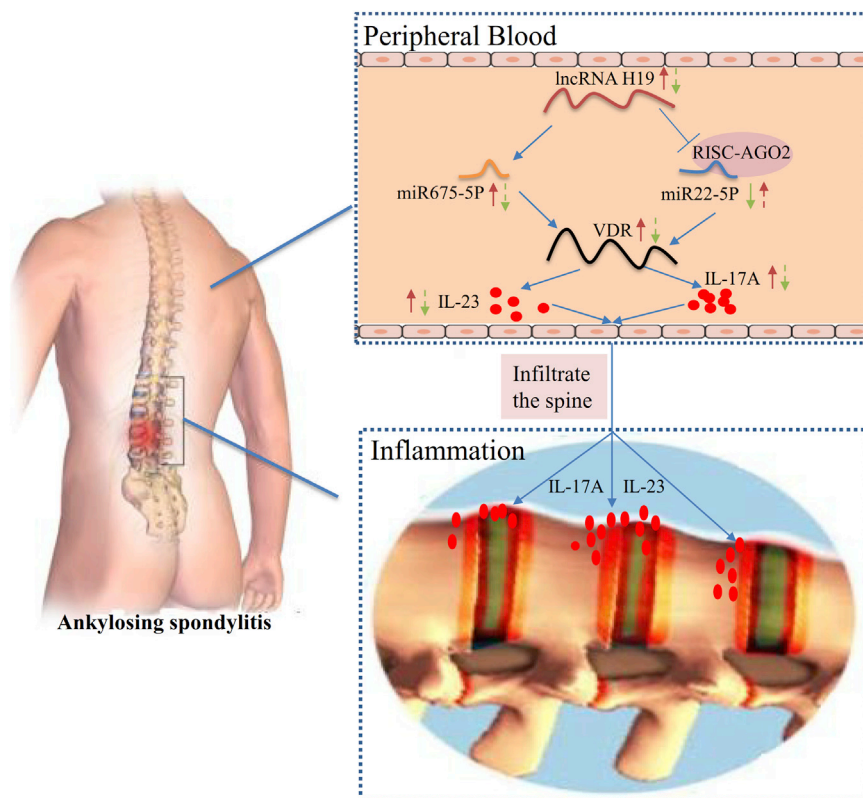


Figure 8. Regulatory Mechanisms of H19 in AS

Dual signaling pathways (H19-miR22-5p-VDR and H19-miR-675-5p-VDR) are involved in the pathogenesis and inflammatory process of AS. One of them is that H19 serves as a ceRNA to sponge miR22-5p by RNA-induced silencing complex (RISC), and another is that H19 directly interacts with miR675-5p. These two pathways affect the expression of VDR and increase the release of IL-17A and IL-23 cytokines, followed by the two cytokines infiltrating the spine and causing or exacerbating the inflammatory response.

qRT-PCR for Detection of miRNA

miRNA was extracted from PBMCs using the miRNeasy Mini Kit (QIAGEN, Germany) and reverse transcribed into cDNA using the miScript II RT kit (QIAGEN, Germany) according to the manufacturer's instructions. The purity and concentration of RNA were determined by UV absorbance at 260 and 280 nm using the NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, DE, USA). qRT-PCR was performed in duplicate on an ABI ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the miScript SYBR Green PCR Kit (QIAGEN, Germany). All reactions were performed in an 8-

strip tube at 95°C for 15 min, followed by 42 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s; melt curve stage was then performed to detect specific amplification at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. RNA relative expression was calculated as FC using the $2^{-\Delta\Delta C_t}$ comparative threshold cycle method, and U6 was used as an internal control. The primers of forward (F) and reverse (R) are listed in Table S3.

Bioinformatics

Online-based RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/>) program was used to predict binding sites for H19, VDR, and TGF- β potentially associated with miR675-5p and miR22-5p. Bioinformatics analyses were carried out by the Gene Pharma Company (Shanghai, China).

qRT-PCR for Detection of lncRNA and mRNA

Total RNA was extracted from PBMCs using the miRNeasy Kit (QIAGEN, Germany) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Japan) according to the recommendations. The purity and concentration of RNA were determined by UV absorbance at 260 and 280 nm using the NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, DE, USA). qRT-PCR reaction was performed in duplicate using the ABI ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR Premix Ex Taq II (Takara Bio, Japan) according to the manufacturer's instructions. All reactions were performed in an 8-strip tube at 95°C for 1 min, followed by 42 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 1 min; melt curve stage was then performed to detect specific amplification at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. RNA relative expression was calculated as FC using the $2^{-\Delta\Delta C_t}$ comparative threshold cycle method, and β -actin was used as an internal control. The primers of forward (F) and reverse (R) are listed in Table S3.

Western Blot

The harvested PBMCs were washed with phosphate buffered saline (PBS) and lysed with a protease inhibitor cocktail in a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Total proteins in the lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bradford, MA, USA). Subsequently, the membranes were blocked with 5% non-fat milk in Tris-Buffered Saline Tween (TBST) (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h at room temperature, followed by incubation with primary antibodies at 4°C for overnight. The primary antibodies were anti-VDR (1:1,000, Cell Signaling Technology, MA, USA), anti-TGF- β (1:1,000, Cell Signaling Technology, MA, USA), and anti- β -actin (1:1,000, Cell Signaling Technology, MA, USA), and anti- β -actin antibody was used as a control. The blots were washed with TBST three times and then incubated with secondary horseradish peroxidase-conjugated antibody (1:5,000 dilution, Biotechnology, Santa Cruz, CA,

USA) for 4 h at room temperature. An enhanced chemo-luminescence detection kit (ECL Advance, UK) was used to expose the film and visualize chemiluminescence. Densitometry of all proteins was normalized against the loading control. Protein bands were calculated using the ImageJ software (NIH, Bethesda, MD, USA).

ELISA

Concentrations of IL-17A, IL-23, and TNF- α in the cell supernatants were measured using human ELISA kits (CUSABIO Life Sciences, MD, USA) according to the manufacturer's recommendations. Briefly, 96-well flat-bottomed high-binding ELISA plates were coated with anti-human IL-17A, IL-23, and TNF- α . Samples and standards were added in plates at 37°C for 2 h and then washed with PBS-T, PBS, and 0.05% Tween-20. After that, they were coated with biotinylated antibody, washed with PBS-T, and avidin-HRP conjugate was added. The above operation was repeated. Colorimetric reactions were incubated with tetramethylbenzidine and terminated with sulfuric acid. Optical density was detected at 450 nm on the microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) and the concentration was calculated using standard curve interpolation.

Transfection

H19 adenovirus (AD-H19), H19 siRNA (Si-H19), and miRNA mimics and inhibitors were purchased from the Gene Pharma Company (Shanghai, China). We designed three siRNA sequences (coded as Si-H19-595, Si-H19-1484, Si-H19-814) to knock down the expression of H19, and AD-H19 was applied to upregulate the expression of H19. In addition, miR22-5p and miR675-5p mimics were used to upregulate the expression of miRNA, miR22-5p, and miR675-5p inhibitors to downregulate their expressions. When cells reached 70%–80% confluence, they were seeded in 12-well plates and were transfected with AD-H19, Si-H19, and miRNA mimics and inhibitors using Lipofectamine 3000 according to the manufacturer's instructions. The sequences of Si-H19, AD-H19, miRNA mimics, and inhibitors are listed in [Table S4](#).

Dual-Luciferase Reporter Assay

Luciferase assay was performed for determining the interaction of miR675-5p and miR22-5p with H19 and VDR. H19 fragment or VDR 3' UTR containing the conserved miR675-5p and miR22-5p binding sites (mutant-type named H19-MUT, VDR-MUT containing a mutation at the predicted miR675-5p and miR22-5p, and wild-type named H19-WT, VDR-WT) was amplified by PCR. The PCR fragment was subcloned into the psiCHECK-2 vector (Promega Corporation, Madison) according to the manufacturer's instructions, and psiCHECK2 empty vector was considered as a NC. Luciferase vectors were co-transfected into HEK293T cells with miR675-5p mimics or miR22-5p mimics (GenePharma) using Lipofectamine 2000. As an internal control, the Renilla luciferase reporters were also co-transfected. After 48 h transfection, dual luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Relative firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis

The normality test of continuous variables was examined by the Kolmogorov-Smirnov test. Student's *t* (bar graph) and Mann-Whitney *U* (scatterplot) tests were used to compare continuous variables in AS and HC groups as appropriate. Pearson's chi-square test was used to compare the between-group difference of categorical variables. ROC curve and AUC were used to assess whether the expression of lncRNA can be a biomarker of AS. Spearman's rank correlation coefficient test was used to explore the correlations between the expression of H19 gene and other molecules (i.e., VDR, TGF- β , IL-17A, IL-23, and TNF- α). All statistical analyses were performed by SPSS 23.0 software (SPSS, Chicago, IL, USA) and diagrams were generated using the GraphPad Prism version 5.01 (GraphPad Software, CA, USA). A two-tailed *p* value <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.11.025>.

AUTHOR CONTRIBUTIONS

F.P. designed the study and obtained funding. X.Z. and S.J. performed experiments. X.Z., G.C., and L.L. wrote and revised the manuscript. Z.P., R.H., Y.Y., S.X., J.Y., Y.M., and M.W. recruited and screened patients. F.P., X.Z., Y.D., and X.G. analyzed and interpreted data. X.H., M.C., and S.G. collected data. S.X. and Z.S. diagnosed patients. All authors participated in the development, review, and approval of the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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