MicroRNA-219c-5p May Participate in Bladder Fibrosis in Multiple Sclerosis Mice by Regulating Fibronectin-1

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Abstract

Background: We found that the bladder of the multiple sclerosis mice was significantly fibrosis. This study aimed to investigate the relationship between Fibronectin-1 (FN1) and bladder fibrosis, as well as microRNAs involved in the regulation of FN1.

Methods: The degree of bladder smooth muscle fibrosis was observed by immunohistochemistry. And we used RT-qPCR (quantitative real-time polymerase chain reaction) and Western blot to identify the expression of FN1 in different grades of fibrosis bladder. It was found by bio information website analysis that miR-1a-3p, miR-219c-5p and miR-3572-3p may prevent inhibition of FN1 synthesis. Thus, miR-1a-3p, miR-219c-5p and miR-3572-3p were overexpressed or knocked down in bladder smooth muscle cells (BMSCs), and the respective transfection efficiency and FN1 knockdown efficiency were detected by RT-qPCR. We discovered that only overexpression and knockdown of miR-219c-5p met the expected results. The dual luciferase reporter assay was used to determine the targeting relationship between miR-219c-5p and FN1. Flow cytometry and Cell Counting Kit 8 (CCK8) experiments confirmed that miR-219c-5p reduced FN1 and affected the biological activity of smooth muscle cells.

Results: As bladder fibrosis worsens, the expression of FN1 is raised, while the expression levels of miR-1a-3p, miR-219c-5p, and miR-3572-3P are decreased. The results of RT-qPCR after transfection showed that only miR-219c-5p was the best to regulate FN1. The results of the dual luciferase reporter gene indicated that miR-219c-5p is targeted to bind to FN1. CCK8 assay and cell cycle assay showed that overexpression of miR-219c-5p inhibited the proliferation of BMSCs, while knockdown of miR-219c-5p promoted the proliferation of BMSCs. Apoptosis assay showed that overexpression of miR-219c-5p promoted apoptosis, while knockdown of miR-219c-5p inhibited apoptosis of BMSCs.

Conclusion: Our findings indicate that up-regulation of FN1 and down-regulation of miR-219c-5p play an important role in the development of bladder fibrosis. And miR-219c-5p may be involved in bladder fibrosis by targeting FN1 expression.

Keywords: Multiple sclerosis; Experimental autoimmune encephalomyelitis; Bladder fibrosis; Fibronectin-1; microRNA-219c-5p

Abbreviations

MS: Multiple Sclerosis; EAE: Experimental Autoimmune Encephalomyelitis; BSMCs: Bladder Smooth Muscle Cells; ECM: Extracellular Matrix; FBS: Fetal Bovine Serum; FN1: Fibronectin-1; qPCR: Quantitative real-time PCR; α-SMA: α-Smooth Muscle Actin; CS: Clinical Score

Introduction

Multiple Sclerosis (MS) is a diffuse inflammatory autoimmune disease marked by a multiplicity of spatial and multiple symptoms of symptoms and signs [1,2]. It is currently believed that MS patients are mainly myelin-specific T cell-mediated autoimmune demyelination diseases. Demyelination of the urinary reflex nerve leads to inconsistency in the movement of the bladder detrusor and sphincter. Therefore, MS patients often have frequent urination, urgency and urinary retention [3-5]. So far, although many reports have investigated the possible mechanisms of the development of lower urinary tract symptoms in MS, the mechanism of bladder fibrosis in the ultimate outcome of bladder dysfunction has not been clearly elucidated [6,7]. The EAE model is currently the most commonly used animal model of MS. Myelin autoantigen specifically activates brain-assisted T cells,
causing inflammatory infiltration of the central nervous system and loss of myelin. Biochemical, immunological and pathological features are very close to MS, so they are widely used to assess the etiology of multiple sclerosis and to find different treatment options [8-10]. Bladder fibrosis is the ultimate outcome of bladder dysfunction [11,12]. Fibrosis is described as the "wrong wound healing process" and there may be many mechanisms involved in its development, but they are all based on the activation of bladder smooth muscle cells and the resulting Extracellular Matrix (ECM) components [13]. Wang et al. [14] first reports that microRNA-101b is induced by hypoxia and represses fibrosis of bladder smooth muscle cells by inhibiting the expression of TGF-$\beta$R1 through TGF-β signaling pathway, and it may be an anti-fibrotic miRNA for therapy. Altuntas CZ et al. [7] first reports that bladder fibrosis and bladder remodeling corresponding to the severity of EAE may be due to increased expression of CTGF and increased connective tissue growth. Antibiotic therapy may be an effective way to control bladder dysfunction, but it has not been well studied [15-17]. Fibronectin-1 is an extracellular macromolecular membrane protein present on the surface of animal cells and is the major non-collagen glycoprotein in the ECM and basement membrane. It is also very abundant in the ECM of various tumors including osteosarcoma, leiomyosarcoma, and gastric cancer [18]. Fibronectin-1, which belongs to the FN family, is involved in a variety of cellular biology processes and plays a role in diseases such as fibrosis [19-21]. We found that the more severe the bladder fibrosis, the more FN1. Then, if the synthesis of FN1 can be inhibited, whether the progression of fibrosis can be alleviated or even retarded.

Numerous studies have shown that miRNAs are involved in many cellular biological processes such as cell invasion, cell migration, cell proliferation, apoptosis, and cell cycle. Therefore, miRNAs may be critical for the diagnosis and treatment of certain diseases [22]. Many miRNAs have been found in the brain spinal cord tissue of EAE mice as a biomarker for MS. Bruinsma et al. [23] report that miR-219 may be a candidate biomarker for MS diagnosis by detecting the loss of miR-219 in the patient’s cerebrospinal fluid. MicroRNAs (miRNAs) are short-chain non-coding RNAs of approximately 22 nucleotides in length, whose primary function is tantamount to participate in biological metabolism by inducing mRNA degradation. Many miRNAs turned out to be involved in the development of fibrosis, including miR-26b-5p and miR-200c [24,25]. Notably, regulation of these miRNA expressions can prevent fibrogenesis in vivo and in vitro, suggesting the importance of miRNAs as potential targets for the treatment of fibrosis diseases [26]. Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) for prediction and FN1 Interacting microRNAs. After comparative analysis, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p all suggested a target for interaction with FN1.

The bladder wall is mainly composed of a mucosal layer, a submucosa, a muscle layer and an outer membrane. Among them, bladder fibrosis is mainly caused by the pathological proliferation of BSMCs in the muscular layer of the bladder wall. A considerably amount of matrix deposition in the cytoplasm of BSMCs affects bladder detrusor compliance and leads to bladder remodeling and fibrosis. Therefore, BSMCs were used as experimental models for our study. To investigate whether microRNAs can target the inhibition of FN1 synthesis and block the progression of bladder fibrosis, we overexpressed and knocked down microRNAs in primary bladder smooth muscle cells (BSMCs) in vitro. The results of RT-qPCR after transfection showed that only miR-219c-5p was the best to regulate FN1. In this study, we established an EAE model to simulate bladder fibrosis in MS patients and found that bladder fibrosis is directly proportional to FN1. Through in vitro experiments on bladder smooth muscle primary cells, we found that miR-219c-5p can inhibit the progression of bladder smooth muscle fibrosis by targeting FN1. This study aimed to investigate the significance of miR-219c-5p expression in bladder fibrosis in vitro and in vivo. In addition, this study determined that miR-219c-5p may directly target the FN1 gene, and the miR-219 c-5p/FN1 signaling pathway may contribute to the diagnosis and treatment of bladder fibrosis.

Materials and Methods

Animals

Forty female C57BL/6j mice (4 weeks to 9 weeks old) were purchased from The Charles River. All mice were kept in the SPF animal room of the Zhengzhou University Animal Experimental Center and exposed to a 12:12 hour light: dark cycle (light from 6 am to 6 pm) in the absence of specific pathogens. The experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Experimental autoimmune encephalomyelitis

To induce chronic EAE, animals were injected subcutaneously with an emulsion of MOG 35-55 in complete Freund’s adjuvant (CFA) (Sigma, USA). Then the pertussis toxin (List Biological Laboratories, Campbell, CA; 0.2 μg per animal) in PBS on days 0, 3, and 7 after immunization [7,27]. The mice were scored daily according to the following signs of nerve damage: 0: no disease; 1: tail weakness and/or moderately awkward gait and/or poor righting ability; 2: tail weakness and/or moderate awkward Gait and/or; 3: hind limb paralysis or mild forelimb weakness (or both); 4: limb paralysis; 5: quadriplegia with urine detained or dying. Another group of animals was injected subcutaneously with CFA emulsion and analyzed as a normal control group.

Prediction of potentially targeted miRNAs that inhibit FN1

Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) for prediction and FN1 Interacting microRNAs. After comparing Context++score, Context ++score percentile, Weighted context ++score, Conserved branch length, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p may interact with FN1.

Mice bladder smooth muscle cells (BSMCs) primary culture

The BSMCs were purchased from Otwo Biotech (HTX3129; Shenzhen, China) and verified by a-SMA immunofluorescence. The BSMCs were cultured in Dulbecco’s Modified Eagle Medium - High Glucose (DMEM-H; Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA) 100 U/ml cyan-streptomycin. Used a-SMA (MA1-06110; Thermo Fisher Scientific, Waltham, MA; 1:200) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077; Abcam, USA; 1:1000) immunofluorescence staining to identify BSMCs. Cells with passage numbers 3 to 10 were used only for all experiments.

Overexpression or knockdown of miRNAs in BSMCs

One day before the transfection, the cells were starved, that is, the cells were seeded in a medium supplemented with 2% FBS at a density of 5 × 10^4 cells/ml. The day of transfection was changed to 10% FBS...
Medium and transfected with miRNA mimic, mimic NC, miRNA inhibitor and inhibitor NC (RIBOBIO, Guangzhou, China), using riboFECT™cP Transfection Kit (RIBOBIO, Guangzhou, China) according to the manufacturer’s instructions.

miR-219c-5p target prediction and luciferase reporter assay

Potential miR-219c-5p binding sites were predicted using online database programs with different algorithms, including Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org). For the luciferase assay, BSMCs (3 × 10³ cells/well) were first seeded in 96-well plates. Then, 1 day later, luciferase reporter plasmid [pmiR-FNI-Wild Type (WT) or pmiR-FNI-Mutant (Mut)] (RIBOBIO, Guangzhou, China) was co-transfected with mir-219c-5p mimic or mimic NC to BSMCs. Luciferase activity was determined 48 h after transfection used the GLOMAX 96 spectrophotometer (Promega Corporation) depending on the manufacturer’s protocol. Firefly luciferase activity was normalized via Renilla luciferase activity.

Quantitative real time polymerase chain reaction

Total RNA was isolated from bladder tissue or BSMCs using TRIzol (Leagene Biotechnology, Beijing, China) according to the manufacturer’s protocol. For RT-qPCR of miRNA, reverse transcription reaction was performed using ReverTra Ace qPCR RT Master Mix (FSQ-101; Japan TOYOBO). However, RT-PCR of FNI was subjected to reverse transcription reaction using ReverTra Ace qPCR RT Master Mix and gDNA Remover (FSQ-301; TOYOBO, Japan). Primers used are listed in Table 1. As described above, the cDNA obtained by reverse transcription was used for real-time PCR. qPCR was performed using a SYBR-Green mix kit (Applied Biosystems; Roche, Inc.) and a 20 μl reaction system at 95°C for 2 seconds, 60°C for 20 sec, and 70°C for 10 sec (40 cycles). Relative miRNA expression levels and other indicators were calculated in triplicates using the 2⁻ΔΔCt method. Expression levels were normalized to β-Actin or U6. The primer sequences are shown in Table 1.

Hematoxylin and Eosin (H&E) and Masson trichrome staining

Mouse bladders were soaked overnight with 4% paraformaldehyde. A 5 μm paraffin sections were made for analysis of bladder fibrosis. According to the protocol, paraffin sections were stained with Hematoxylin and Eosin (H&E) and Masson. The amount of collagen, the amount of smooth muscle cells, and the ratio of collagen to smooth muscle cells was calculated by using an image analysis system (Image J 1.80). The ratio of collagen to smooth muscle cells was used to quantify the degree of fibrosis in bladder tissue.

Immunohistochemistry

Immunohistochemical (IHC) staining of Streptavidin-Peroxidase (SP) was used to detect the expression of FNI in bladder tissue. After sectioning, conventional dewaxing, gradient ethanol hydration, and antigen retrieval were performed in sequence. Subsequently, it was incubated with 3% hydrogen peroxide for 20 min, serum blocked for 10 min, and primary antibody anti-rabbit anti-mouse FNI antibody (ab45688; Abcam, USA; 1:200) was incubated overnight at 4°C. They were then incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (ab205718; Abcam, USA; 1:2000) for 30 min and finally stained with DAB (Sigma, USA). After counterstaining with hematoxylin, all slides were hydrated, clarified, at 4°C. They were then incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (ab205718; Abcam, USA; 1:2000) for 30 min and finally stained with DAB (Sigma, USA). After counterstaining with hematoxylin, all slides were hydrated, clarified, fixed and observed, and the cell positive index was counted.

Cell proliferation assay

Cell proliferation was tested using Cell Counting Kit 8 (CCK8) (CK04; DOJINDO, Japan; 10 μl). The BSMCs to be transfected were inoculated into 96-well plates. Each well was inoculated with about 1000 cells, and 6 wells were repeated for each group. After 24 h, 48 h, 72 h and 96 h of transfection, 10 μl of CCK8 was added to each well and incubated for 1 h before being tested on the machine. Sample absorbance was read at 450 nm on a microplate reader (SpectraMax-M5, Sunnyvale, CA).

Table 1: List of primers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>F-AAATGGTGCTGAGGCTTTTGATAC</td>
</tr>
<tr>
<td>U6</td>
<td>F-GCTGCTTCCGTCGAAGGCACA</td>
</tr>
<tr>
<td>FN1</td>
<td>F-ACCCGTTTTCATCACAAAGAG</td>
</tr>
<tr>
<td>miR-1a-3p</td>
<td>UAUGUUAUGAGAAAGUAUGAGGU</td>
</tr>
<tr>
<td>miR-219c-5p</td>
<td>GCUCUCAGCGACCCUGACG</td>
</tr>
<tr>
<td>miR-3572-3p</td>
<td>GACCCCCUCUCUCGUGUACAU</td>
</tr>
</tbody>
</table>

FN1: Fibronectin-1; F: Forward; R: Reverse.
Flow cytometry

After 48 h of transfection, cells were treated with 0.25% trypsin and were harvested. Approximately 1 × 10^6 cells were counted, fixed with 75% cold ethanol (Solarbio Life Sciences, Shanghai, China) for 4 h, and then treated with 0.5% Triton-X100 for 10 min. Triton-X100 was removed by centrifugation and stained with 200 μl Propidium Iodide (PI) (BD, USA) at room temperature for 30 min in the dark. A flow cytometer (ACCURI C6 PLUS, BD, USA) was used to analyze the cell cycle and the experiments were repeated three times.

Similarly, cells were treated with 0.25% trypsin without Ethylenediaminetetraacetic acid. About 1 × 10^6 cells were counted, and 10 μl of each of Annexin V-Fluorescein Isothiocyanate (FITC) (BD, USA) and PI (BD, USA) was added and incubated at room temperature for 15 min in the dark. The rate of apoptosis (percentage of apoptosis cells relative to the total number of cells) was estimated by flow cytometry. Representative results for three independent experiments are shown.

Western blots assay

An equal amount of each protein sample (20 μg) was separated using a 12% Sodium Dodeyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) at a voltage of 120V. The membrane was transferred to a Polypropylene Difluoride (PVDF) membrane by constant flow of 400 mA for 5 h under ice bath conditions. The PVDF membrane was immerssured for 30 min at room temperature using 5% skim milk powder for blocking non-specific bands. The PVDF membrane was incubated with a primary antibody, rabbit anti-mouse FN1 (ab45688; Abcam, USA; 1:10000), and β-Actin (AC026; ABclonal, Wuhan, China; 1:10000) at 4°C overnight. After washing once with TBS and 3 times with TBST, the PVDF membrane was incubated with goat anti-rabbit anti-IgG secondary antibody (AS003; ABclonal, Wuhan, China; 1:10000) conjugated to specific horseradish peroxidase for 1 hour at room temperature. The PVDF membrane was washed 5 times with TBST and then detected using an Enhanced Chemiluminescence (ECL) system. Grayscale analysis of protein bands was performed using Image-J software (National Institutes of Health, Bethesda, MD, USA) and normalized to the gray value of β-Actin. This experiment was replicated three times.

Statistical analysis

All data were from three independent replicates and are shown as Mean (M) ± Standard Deviation (SD). A comparison between the two sets of samples was performed using Student’s t test. One-way ANOVA or two-way ANOVA was used for comparison between single factors or two factor multiple groups. P<0.05 was deemed to represent a statistically significant difference. Statistical analysis was performed using SPSS software (SPSS for Windows 21.0; SPSS Inc., Chicago, IL, USA).

Results

Bladder dysfunction in EAE mice

On the 10th day after immunization, EAE mice were observed to show signs of neurological deficit, while the normal control mice (immunized with CFA alone) were normal. And we found that the symptoms of EAE mice were not persistently aggravated, but rather a cycle of sustained remission-relapse. However, the mice in the remission phase were not completely normal and still had symptoms of weakness in the tail. Figure 1A shows the mean Clinical Symptom score (CS) from day 0 to day 50. The Normal Control mice (NC) showed no damage throughout the time period. At 50 days post-immunization, the bladder-body weight ratio of the EAE model group (CS 1/2/3 group) was significantly higher than that of the NC group (Figure 1B), whereas the mean body weight was not significantly different in NC mice and EAE mice (Table 2). HE and Masson staining showed that the CS3 group was significantly more fibrous than the NC group (Figure 1C). Therefore, the higher the CS score of EAE mice, the more severe the bladder fibrosis.

Expression of FN1 in bladder tissues

We found a potential correlation between bladder fibrosis and FN1 by detecting protein and mRNA expression levels in varying degrees of fibrosis bladder. As the clinical symptoms of EAE model mice deteriorated, the expression level of FN1 increased significantly. We detected an increase in FN1 expression by immunohistochemistry, Western blot analysis and RT-qPCR as bladder fibrosis was aggravated (Figure 2).

Predict miRNAs acting on FN1 and detect miRNA expression in bladders with different degrees of fibrosis

Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) for prediction and FN1 Interacting microRNAs. After comparing Context++score, Context++score percentile, Weighted context++score, Conserved branch length, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p may interact with FN1 (Figure 3A). The expression levels of miRNAs in the bladder of different scores were detected by RT-qPCR (Figure 3B-3D). We found that the expression levels of miR-1a-3p, miR-219c-5p and miR-3572-3p decreased with the increase of bladder fibrosis. That is, the opposite of the FN1 trend.

Identification of bladder smooth muscle cells and miRNA transfection

Bladder Smooth Muscle Cells (BSMCs) were identified by

<table>
<thead>
<tr>
<th>Variable</th>
<th>EAE Score</th>
<th>F Value</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Body weight</td>
<td>27.99 ± 0.56</td>
<td>27.91 ± 0.78</td>
<td>25.61 ± 1.05</td>
</tr>
<tr>
<td>Bladder weight to total body weight, %</td>
<td>0.17 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>FN1</td>
<td>1.00 ± 0.10</td>
<td>1.23 ± 0.70</td>
<td>1.59 ± 0.14</td>
</tr>
<tr>
<td>miR-1a-3p</td>
<td>3.40 ± 0.53</td>
<td>2.73 ± 0.25</td>
<td>1.93 ± 0.40</td>
</tr>
<tr>
<td>miR-219c-5p</td>
<td>3.10 ± 0.10</td>
<td>1.73 ± 0.08</td>
<td>1.23 ± 0.06</td>
</tr>
<tr>
<td>miR-3572-3p</td>
<td>4.50 ± 0.50</td>
<td>3.53 ± 0.55</td>
<td>2.03 ± 0.25</td>
</tr>
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</table>

Values are means ± SE; n=6 mice/group. P<0.05 by ANOVA among four groups; Expression levels were normalized to β-Actin or U6.
immunofluorescence using α-SMA (Figure 4A). The transfection efficiency of miR-1a-3p, miR-219c-5p, and miR-3572-3p was detected by RT-qPCR (Figure 4B). The findings were from three different experiments. Data are expressed as Mean (M) ± Standard Deviation (SD).

miR-219c-5p binding to FN1

The bio information prediction websites such as, Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) were used to infer the potential targeting relationship between miR-1a-3p, miR-219c-5p, and miR-3572-3p on FN1 was detected by RT-qPCR (Figure 4C). We found that only miR-219c-5p achieved the expected results in the case of no difference in transfection efficiency between miR-1a-3p, miR-219c-5p, and miR-3572-3p. That is, FN1 was decreased when overexpressing miR-219c-5p and FN1 was increased when miR-219c-5p was knocked down. In summary, miR-219c-5p is most likely one of the miRNAs acting on FN1.

miR-219c-5p targeted FN1 for suppressing BSMC proliferation

The effect of miR-219c-5p on cell proliferation in BSMCs is shown in Figure 5B. There was no significant difference in the survival rate of BSMCs cells between the blank and NC groups (all P>0.05). Compared with the blank and NC groups, the viability of the BSMCs in the miR-219c-5p group decreased (P<0.05), whereas cell viability in the miR-219c-5p inhibitor group increased (P<0.05).

Effect of miR-219c-5p on the BSMC cycle

Compared with the blank and NC groups, the number of BMSCs in the G1 phase of the miR-219c-5p group increased, but decreased in the S/G2 phase, while the number of cells in the G1 phase of the miR-219c-5p inhibition group decreased, but increased in the S/G2 phase (P<0.05 in all cases) (Figure 6A). These data indicate that miR-219c-5p can inhibit the cell cycle by regulating the expression of the FN1 gene and thereby arresting BSMCs in the G1 phase.

Effect of miR-219c-5p on cell apoptosis in BSMCs

The apoptosis rate of the miR-219c-5p mock group increased significantly compared with the blank and NC groups (P<0.05). However, the apoptosis rate of the miR-219c-5p-inhibited group reduced significantly compared with the blank and NC groups (P<0.05) (Figure 6B). These results indicate that miR-219c-5p can promote BSMC apoptosis.

Discussion

In this study, we found that bladder fibrosis in EAE mice was aggravated with clinical symptoms. Moreover, we found that bladder fibrosis aggravation positively correlates with FN1. Therefore, we speculated whether the synthesis of FN1 can be inhibited by certain miRNA targeting, thereby blocking the progression of bladder
fibrosis. Three sites, such as Target Scan (http://www.targetscan.org), were used to predict microRNAs that interact with FNI. After comparative analysis, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p all suggested a target for interaction with FNI. By overexpressing or knocking down miR-1a-3p, miR-219c-5p and miR-3572-3p in bladder smooth muscle in vitro, we found that only miR-219c-5p targeted FNI was better. The luciferase reporter assay confirmed that miR-219c-5p can target the inhibition of FNI synthesis. Moreover, CCK8 and flow functional assays also confirmed that miR-219c-5p can regulate the function of BSMCs by targeting the regulation of FNI synthesis. Urinary dysfunction such as frequent urination, urgency, urinary retention is common problems in patients with MS. The treatment of MS patients with bladder dysfunction remains a clinically difficult problem because there is at present no effective treatment. Altuntas et al. [7] first suggested that the EAE remains a clinically difficult problem because there is at present no effective treatment. Altuntas et al. [7] first suggested that the EAE is abnormally expressed in multifactorial Hirschsprung's disease and whether miRNA is abnormally expressed in multifactorial Hirschsprung's disease and whether miRNA may involve many mechanisms, but activation of myofibroblasts and subsequent production of excess extracellular groups are essential for fibrosis. Similarly, bladder fibrosis is thought to be involved in the activation of bladder smooth muscle cells and their accumulation of excess extracellular matrix. Some studies have shown that miRNAs are involved in smooth muscle cell function, including cell proliferation, cell cycle, and apoptosis [24,30,31]. There is growing evidence that many miRNAs play key regulatory roles in bladder fibrosis. Duan et al. [32] revealed a novel miR-133 regulatory factor that targets the TGF-β-Smad3 signaling pathway to modulate TGF-β1-induced changes in a BSMC phenotype. A novel anti-fibrotic function of miR-133 is proposed, which represents a potential target for diagnosis and treatment strategies of bladder fibrosis.

FNI is a high molecular weight glycoprotein in the extracellular matrix with complex biological functions as a core component. Fibroblasts, vascular endothelial cells, hepatocytes, vascular smooth muscle cells, and other cell types secrete these functional proteins, which regulate cell adhesion, proliferation, and differentiation, maintenance of cell morphology, promotion of cell migration, ion exchange, signal transduction, and other functions. Numerous studies have reported the regulation of FNI-mediated fibrosis by miRNAs. Gunadi et al. [33] reported that miRNA-206 targeting FNI is abnormally expressed in multifactorial Hirschsprung's disease and can be used as a potential targeting molecule.

Numerous studies have shown that miRNAs are involved in the tissue fibrosis process. Jing et al. [34] reported that miR-27a can target AMP-activated protein kinase alpha 2 catalytic subunit and activate TGF-β1 and Smad3 signaling pathways, thereby reducing detrusor fibrosis in streptozotocin-induced diabetic rats. Moon et al. [35] reported that FNI is an important functional protein in the development of skin fibers. Therefore, to explore whether FNI is involved in the development of bladder fibrosis, and whether miRNA can target FNI to regulate the progression of bladder fibrosis. Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) prediction found miR-1a-3p, miR-219c-5p and miR-3572-3p may interact with FNI.
Taken together, this study reported that miR-219c-5p is better targeted to regulate FN1 than miR-1a-3p and miR-3572-3p. This study demonstrated that miR-219c-5p blocks fibrosis of bladder smooth muscle cells by targeting inhibition of FN1 expression. However, the experimental deficiency is that the effect of miR-219c-5p regulating FN1 on bladder fibrosis has not been demonstrated in animals.

**Conclusion**

In conclusion, this study confirmed that FN1 and miR-219c-5p are associated with bladder fibrosis in MS mice. It also shows that miR-219c-5p can regulate the proliferation and apoptosis of bladder smooth muscle cells by targeting FN1. Thus, miR-219c-5p and FN1 are potentially promising biomarkers for the diagnosis and treatment of bladder fibrosis.

**Declarations**

**Ethical approval and consent to participate**

The experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-202).

**Availability of supporting data**

The datasets generated and analysed during the current study are available the Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org), miRBase (http://www.mirbase.org).
Funding

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Authors’ Contribution

JY and ZJ have made significant contributions to the concept and design. CG and TW collected data. The data was analyzed and interpreted by YD and PL. BL participated in the drafting of the manuscript. JY and SH participated in critical revisions of important knowledge content. All authors have finally approved the version to be released and agree to be responsible for all aspects of the work.

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