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Original Article

Triamcinolone acetonide combined with 5-fluorouracil suppresses urethral scar fibroblasts autophagy and fibrosis by increasing miR-192-5p expression

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Abstract: Urethral stricture is one of the common diseases in urology. It can lead to obstructive voiding dysfunction and may cause long-term damage to the entire urinary tract. Here, we investigated the effect of combined use of 5-fluorouracil (5-FU) and triamcinolone acetonide (TA) in improving urethral stricture. We established urethral stricture *in vivo* and *in vitro* model. The role of TA combined with 5-FU treatment in scar tissue and fibroblast cells were examined by RT-PCR, Western blot and immunohistochemical methods. The function of miRNA in improving urethral stricture by TA combined with 5-FU treatment were further investigated. We found that TA combined with 5-FU treatment obviously prevent urethral fibrosis *in vivo* as well as *in vitro*. MiR-192-5p level was downregulated in urethral stricture tissue and urethral tissue fibroblast, TA combined with 5-FU treatment rescue the expression of miR-192-5p. The improvement of urethral fibrosis by TA combined with 5-FU treatment was blocked by miR-192-5p inhibitor. miR-192-5p mediated the improvement of urethral scar by triamcinolone acetonide combined with 5-FU by directly targeting ATG7, which is marker gene of autophagy. Our data demonstrated that TA combined with 5-FU suppresses urethral scar fibroblasts autophagy and fibrosis by increasing miR-192-5p expression, thus offering a new strategies and target for Urethral stricture.

Keywords: Urethral stricture, triamcinolone acetonide, 5-fluorouracil, miR-192-5p, autophagy

Introduction

Urethral stricture, a kind of keloids, is caused by fibrosis of urethral mucosa and surrounding cavernous body [1]. Although great progress has been made in modern medicine and surgery, the therapy of urethral stricture still remains very difficult [2]. With the continuous advancement of surgical technology, the gradual use of allogeneic materials such as oral mucosa, colonic mucosa and acellular matrix, the success rate of urethral stricture treatment has been significantly improved [3]. Urethral dilation and internal urethrotomy, however, these managements have a high failure rate (90%) and a poor long-term outcome [4]. Moreover, the performance of current surgical such as direct end-to-end anastomosis or two-stage urethroplasty is frequently accompanied by subsequent chronic inflammation and tissue fibrosis, which eventually lead to the formation of secondary inflammation [5]. Although many anti-fibrotic drugs, such as triamcinolone acetonide (TA), halofuginone, mitomycin-C and glucocorticoids have been used to treat urethral strictures, they have proved to be of little benefit [6-9]. None of these drugs have been proved to be with sufficient therapeutic benefit.

The International Advisory Panel on Scar Management suggested that intradermal steroid injection for the treatment of keloids and hypertrophic scars [10]. TA, a long-acting intradermal steroid has become the most popular drug, have achieved good therapeutic effects in the treatment of hyperplastic paralysis of the skin [11]. The clinical treatment efficacy ranging from 50% to 100% and the recurrence rate ranging from 9% to 50%. Previous study have shown that TA induces a significantly decrease of alpha-1-antitrypsin and alpha-2-macroglobulin, which is upregulated in keloidal tissue [12]. 5-flurouracil (5-FU) is an analog of uracil, which acts as an antimetabolite after being converted into an effective fluorouracil deoxynucleotide within the cell and can interfere with DNA syn-

thesis [13]. Without biosynthetic structural elements, rapid proliferation of cells such as fibroblasts are blocked, promoting scar degradation. 5-FU was first introduced in the treatment of keloid in 1999 [14]. Thereafter, several researchers have clarified its efficacy in the treatment of keloids. It has been proved that combined application of TA combined with 5-FU treatment has quick reaction and fewer side effects on scar formation [15]. However, the molecular mechanism of 5-FU/TA in the treatment of urethral scar remains unclear.

MiRNAs are endogenous non-coding small RNAs 18-22 nucleotides. In recent years, studies on the regulation mechanism of gene expression have shown that miRNAs are involved in regulating the occurrence and development of skin diseases and wound repair, regulating the formation of scars, and there are differences in expression between hypertrophic scars and keloids [16, 17]. In the current study, our data shown that combining TA with 5-FU could improve the fibrosis of urethral scar fibroblasts and our data shown that miR-192-5p level was decreased in urethral stricture tissue as well as urethral tissue fibroblast, TA combined with 5-FU treatment rescue the miR-192-5p expression. Predicting the corresponding target genes or target proteins of miR-192-5p and upregulating or downregulating the corresponding signal molecules can realize the regulation of the occurrence and development of urethral scar at the gene level and provide a new platform and ideas for the treatment of urethral scar. Further study shown that ATG7 was the directly target gene of miR-192-5p and autophagy mediated the pro-fibrotic effects of miR-192-5p.

Materials and methods

Urethral scar rat model

Adult male SD rats (300-350 g; n=5 of each group) were selected from SLAC Laboratory Animal Co., Ltd (Shanghai, China) and kept at a constant temperature with adequate food and water under the light/dark cycle (12 h/12 h). Related rat experiments were approved by Ethics Committees of the Tongji Hospital, Tongji University School of Medicine. In brief, each rat was anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/kg). To facilitate urethral exposure and prevent urethral injury, a lubricated urinary catheter (polyethylene tube, 0.61 mm in diameter [equal to 1.8 French]) was gently inserted into the urethra. A small penoscrotal incision was created and the rat urethra was meticulously dissected. 25 rats were randomized into five equal groups: 1) In the control group, rats in this group were not treated; 2) The urethra scar group, 0.4 ml saline solution was injected into the urethra of urethra scar rats at the 3 and 9 o'clock positions with a 30-gauge needle; 3) The urethra scar+5-FU treatment group, 5-FU (Nantong Jinghua Pharmaceutical Co., Ltd., Jiangsu, China) of 2.5 mg in 0.2 mL saline solution was injected into the urethra at the 3 and 9 o'clock positions with a 30-gauge needle once a week for 4 weeks; 4) The urethra scar+ TA treatment group, TA (Zhejiang Xianju Pharmaceutical Co Ltd, Zhejiang, China) of 1 mg in 0.2 mL saline solution was injected into the urethra at the 3 and 9 o'clock positions with a 30-gauge needle once a week for 4 weeks; 5) The urethra scar+5-FU/TA treatment group, 5-FU/TA of 3.5 mg (5-FU, 2.5 mg; TA, 1 mg) in 0.4 mL saline solution was injected into the urethra at the 3 and 9 o'clock positions with a 30-gauge needle once a week for 4 weeks.

Cell culture and drug treatment

Fibroblasts derived from urethral scar tissue were separated, cultured and detected according to the previous description [18]. In brief, the fresh urethral scar tissue was washed with PBS, the residual plasma was removed, cut into 1-mm^3 pieces, and inoculated in tissue culture dish for preservation. Cells was isolated by trypsin and further cultured in DMEM with 10% FBS at 37°C with 5% CO_2. Cells were treated with TA (20 mM) and 5-FU (1 mg/ml) for 72 h.

Overexpression and inhibition of miR-192-5p

miRNA mimic and inhibitor were used to increase or inhibit miR-192 expression as the manufacturer's protocols, respectively. In brief, when cells grew to 60-70% confluence, the compound of 100 nM of miR-192 mimic, miR-192 inhibitor and their corresponding negative control (NC mimic or NC inhibitor) (Shanghai Genechem Co., LTD., Shanghai, China) with 5 μl Lipofectamine 3000 (Invitrogen) were added to the cellular in Opti-MEM medium. The miR-192 expression in cells was further verified by RT-PCR methods.

Table 1. Primers used in this manuscript

Gene	Primer sequence
miR-192-5p (Forward)	5'-GGACTTTCTTCATTC ACACCG-3'
miR-192-5p (Reverse)	5'-GACCACTGAGGTTAGAGCCA-3'
GAPDH (Forward)	5'-TGCTGAGTATGTCGTGGAGTCTA-3'
GAPDH (Reverse)	5'-AGTGGGAGTTGCTGTTGAAATC-3'

ATG7 overexpression

For ATG7 overexpression, ATG7 mRNA sequence was synthesized and subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen), and the empty pcDNA3.1 vector served as a negative control. Plasmid transfection was performed by using Lipofectamine 3000 reagent following the manufacturer's protocol.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was separated from tissues or cells by Trizol kit (Takara, Japan). cDNA was generated by an iScript Advanced cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-PCR was performed via SYBR Green PCR Master-Mix (Solarbio, Beijing, China) on a Step One plus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) under the reaction system and reaction procedure provided in the manual. Relevant primer sequences are shown in the **Table 1**. The $2^{-\Delta\Delta Ct}$ method was used to determine the fold changes of each gene. GAPDH was used as an internal control.

Immunohistochemical

Freshly urethral scar tissue, drug-treated urethral scar tissue and normal urethra tissue were fixed in 4% paraformaldehyde in the 4°C environment for one day. After that, all tissues were dehydrated in embedding box and embedded using liquid paraffin as the instruction's procedure (Sigma-Aldrich, St. Louis, MO, USA). The sections (4 μm) were prepared through a slicer and baked on the slide warmers for 30 min.

The sections were placed in a wet box, in which a small amount of distilled water was added, plus 3% hydrogen peroxide, and incubated for 10 minutes. Washing in PBS and distilled water 3 times each, 3 minutes each time. Immuno-histochemistry were carried out with anti-α-SMA antibody (0.034 μg/ml; ab7817, Abcam), anti-COL1 antibody (1:200; ab254113, Abcam), and anti-LC3B antibody (1:400; ab48394, Abcam) according to instruction's procedures. 100 μL DAB solution (Sigma) was added to each section, re-dyed with hematoxylin for 1-2 min.

Western blot

Total protein in the cells was extracted with lysis kit (Thermo Fisher Scientific). The BCA kit (Beyotime, Shanghai, China) was applied to determine the protein concentration of the sample. 10% SDS-PAGE gels were used to separate the same amount of protein, and then transferred to PVDF membranes. Blocking in milk (5%, non-fat), the PVDF membranes were incubated with anti-Collagen I-antibody (1:1000; ab254113, Abcam), anti-α-SMA-antibody (1:5000; ab32575, Abcam), anti-LC3 I-antibody (2 μg/ml; ab62721, Abcam), anti-LC3-II-antibody (2 μg/ml; ab62721, Abcam) or anti-ATG7-antibody (1 μg/ml; ab53255, Abcam) overnight at 4°C. After that, the PVDF membranes were washed with PBST followed by an incubation with Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (1:10000; 31460, Invitrogen) for 2 h at room temperature. The bands on the membrane were visualized with the ECL reagent (Solarbio).

Luciferase reporter assay

The full length of the human ATG7 3'-UTR were cloned downstream to the firefly luciferase coding region. Cells were plated into 12-well plates for 16 h. The luciferase reporter plasmid was transfected when the cell density reached about 80%. Lipofectamine 2000 (Invitrogen) was used to co-transfect miR-192-5p mimics or NC (50 nM) and reporter plasmid (100 ng/ml) into cells. After 36 h, the luciferase activity was assayed by the Dual-Luciferase Assay kit (Promega Biotech Co., Ltd., Madison, WI, USA).

Statistical analysis

The data presented were mean ± SD at least three independent experiments. Differences between two groups or more than two groups were carried out via the student's *t*-test or one-way ANOVA, respectively. Significance level was set as P less than 0.05. All data were performed with SPSS statistical package (version 17.0; SPSS, Chicago, IL, USA).

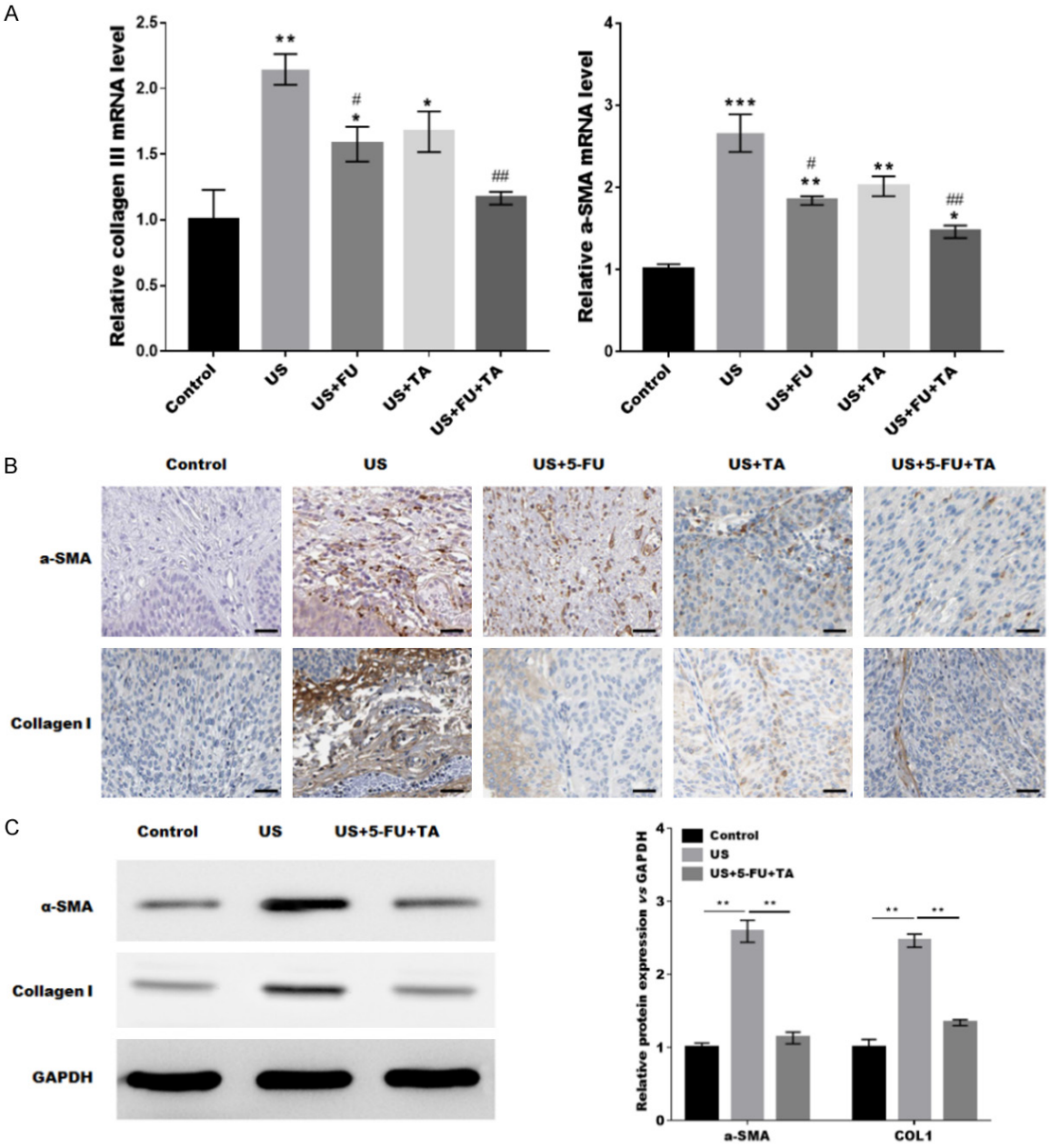


Figure 1. TA combined with 5-FU suppresses urethral fibrosis in vivo. A. RT-PCR analysis of α-SMA and collagen I mRNA expression in urethral tissues of urethral scar rats treated with 5-FU (2.5 mg, n=5), TA (1 mg, n=5) or 5-FU/TA (2.5 mg 5-FU+1 mg TAC, n=5). B. The expression of α-SMA and collagen I was detected by IHC in urethral tissues of urethral scar rats treated with 5-FU, TA or 5-FU/TA. Scale bars =50 μm for IHC staining. C. Western blot analysis for α-SMA and collagen I protein level in urethral tissues of urethral scar rats treated with 5-FU, TA or 5-FU/TA. **P*<0.05, ***P*<0.01, ****P*<0.001. RT-PCR, Reverse Transcription-Polymerase Chain Reaction; TA, Triamcinolone acetonide; 5-FU, 5-fluorouracil; 5-FU/TA, TA combined with 5-FU treatment; IHC, Immunohistochemistry.

Results

TA combined with 5-FU suppresses urethral fibrosis in vivo

To verify the effect of TA combined with 5-FU in urethral scar, we performed RT-PCR, Western blot and immunohistochemistry assays to detect α-SMA and collagen I. As shown in **Figure 1A**, 5-FU treatment reduced α-SMA and collagen I mRNA expression level, while 5-FU treatment reduced α-SMA and collagen I mRNA expression level, but there was no significant difference. Combined with 5-FU treatment obviously reduced α-SMA and collagen I mRNA

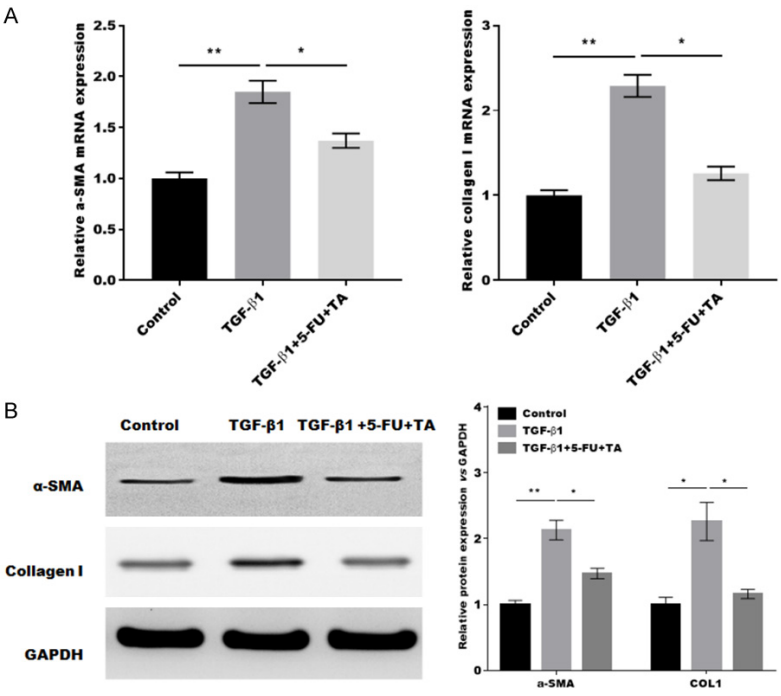


Figure 2. TA combined with 5-FU suppresses urethral fibrosis in vitro. A. RT-PCR analysis of α-SMA and collagen I mRNA expression in primary urethral scar fibroblasts isolated from urethral scar tissues and induced by TGF-β1 (10 ng/ml for 24 h). B. Western blot analysis for α-SMA and collagen I protein level in primary urethral scar fibroblasts isolated from urethral scar tissues and induced by TGF-β1 (10 ng/ml for 24 h). RT-PCR, Reverse Transcription-Polymerase Chain Reaction. **P*<0.05, ***P*<0.01.

expression level. Similar to the RT-PCR results, Immunohistochemical staining showed that α-SMA and collagen I expression were obviously decreased by TA combined with 5-FU treatment, compared with urethral scar model group (**Figure 1B**). Analogously, Western blot data showed that the protein expression of α-SMA and collagen I were remarkably reduced in TA combined with 5-FU group, contrasted with urethral scar model group (**Figure 1C**). * vs Control, # vs US group.

TA combined with 5-FU suppresses urethral fibrosis in vitro

We isolated and cultured primary urethral scar fibroblasts from urethral scar tissues and investigated the function of combined use of TA and 5-FU *in vitro*. TGF-β1 has the ability to promote fibrosis. We established the cell model of urethral scar fibroblasts fibrosis induced by TGF-β1 (10 ng/ml for 24 h). As shown in **Figure 2A**, α-SMA and collagen I mRNA level were significantly boosted after TGF-β1 stimulation in urethral scar fibroblasts, and these effects were blocked by TA combined with 5-FU treatment. Similar to the RT-PCR results, Western blot data showed that the expressions of α-SMA and collagen I were decreased through TA combined with 5-FU treatment, contrasted with TGF-β1 stimulated alone group (**Figure 2B**).

miR-192-5p is involved in the function of TA combined with 5-FU suppresses urethral fibrosis

Fifteen urethral stricture related miRNAs were selected from microRNA expression profiles of scar and normal tissue from patients [16]. RT-PCR array was performed to verify the expression of 15 miRNAs in control, urethral scar model and TA combined with 5-FU treated urethral scar model. As shown in **Figure 3A**, 3 abnormally expressed miRNAs were detected, with 1 up-regulated (miR-128-3p) and 2 down-regulated (miR-192-5p and miR-92a-3p) in urethral scar model group compared with control group. Further data suggested that only miR-192-5p was rescued in TA combined with 5-FU treated urethral scar model (**Figure 3B-D**). In vitro data also found that miR-192-5p was rescued in TA combined with 5-FU (**Figure 3E** and **3F**).

Selective inhibition of miR-192 blocked the improvement of TA combined with 5-FU on urethral fibrosis

To evaluate the functional significance of miR-192 in urethral fibrosis, we explored the effect of miR-192 in urethral scar cell model induced by TGF-β1. We knockdown miR-192 in urethral fibroblast with or without TA combined with 5-FU treatment. As shown in the **Figure 4A**, TA combined with 5-FU treatment decreased the expression level of α-SMA and collagen I induced by TGF-β1, whereas miR-192-5p inhibition inhibited this effect. Next, the protein level of α-SMA as well as collagen I was detected through Western blot. As expected, the result was similar to RT-PCR data (**Figure 4B**). These

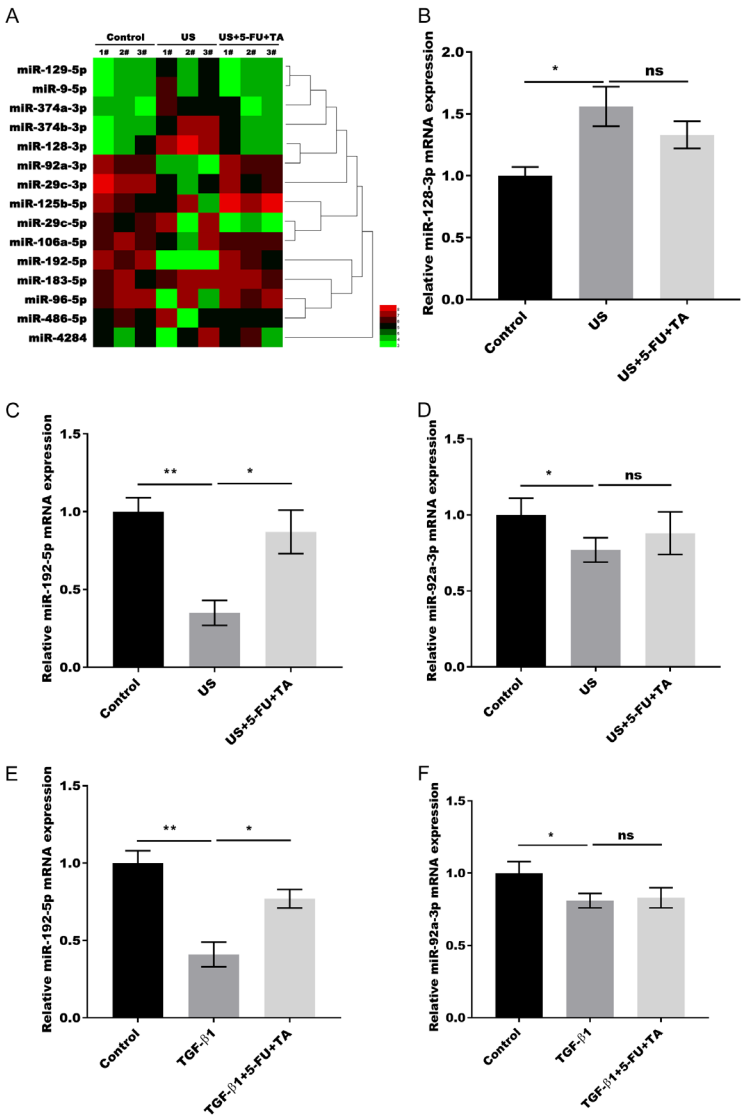


Figure 3. miR-192-5p is involved in the function of TA combined with 5-FU suppresses urethral fibrosis. (A) Relative expression of fifteen miRNAs in rat urethral tissues of control rats, urethral scar rats or urethral scar+5FU/TA rats were detected by RT-PCR array. (B-D) The mRNA expression of miR-128-3p (B), miR-192-5p (C) and miR-92a-3p (D) in rat urethral tissues of control rats, urethral scar rats or urethral scar+5FU/TA rats were confirmed by RT-PCR. (E, F) The mRNA expression of miR-192-5p (E) and miR-92a-3p (F) in primary urethral scar fibroblasts isolated from urethral scar tissues treated with TGF-β1 in the absence or presence of 5-FU/TA were confirmed by RT-PCR. 5-FU/TA, TA combined with 5-FU treatment. **P*<0.05, ***P*<0.01, ns, no significant.

results indicated that miR-192-5p mediated the improvement of urethral scar by TA combined with 5-FU.

miR-192-5p inhibits autophagy by directly targeting ATG7

To clarify the mechanism of miR-192-5p mediated the improvement of urethral scar by TA combined with 5-FU. The target of miR-192-5p was predicted by three bioinformatics prediction databases targetscan (http://www.targetscan.org/), miRDB (http://mirdb.org/) and TarBase (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8/index) (miRNA target gene prediction databases). 6 common targets were found and further confirmed in control, urethral scar model and TA combined with 5-FU treated urethral scar model by RT-PCR (**Figure 5A** and **5B**). As shown in **Figure 5B**, only ATG7 was upregulated in urethral scar and downregulated by TA combined with 5-FU treatment, this trend is exactly opposite to miR-192-5p. Therefore, we chose ATG7 as the target for miR-192-5p. We found that ATG7 3'-UTR contains putative binding sites for miR-192-5p (**Figure 5C**), Hence, we constructed the wild type and mutation luciferase reporter of miR-192-5p potential binding site in ATG7 mRNA 3'UTR. The above results indicated that miR-192-5p inhibited the reporter activity of the transcript containing wild-type 3'-UTR of ATG7, suggesting the direct regulation of miR-192 on ATG7 (**Figure 5D**). Furthermore, Western blot detection showed that treatment with TGF-β alone increased the ATG7 and LC3 expression, whereas overexpression of miR-192-5p decreased TGF-β-induced ATG7 and LC3 expression (**Figure 5E**).

To confirm the autophagy is involving in the fibrosis, urethral fibroblast cells were treated with TGF-β1 either in the absence or presence of 3-MA, then the autophagy and fibrosis Marker was detected by western blot. As shown in **Figure 6A**, the inhibition of autophagy obviously decreased the cell fibrosis. To further confirm that miR-192-5p inhibits autophagy by directly targeting ATG7, urethral fibroblast cells were treated with TGF-β1 and overexpressed with miR-192-5p, either

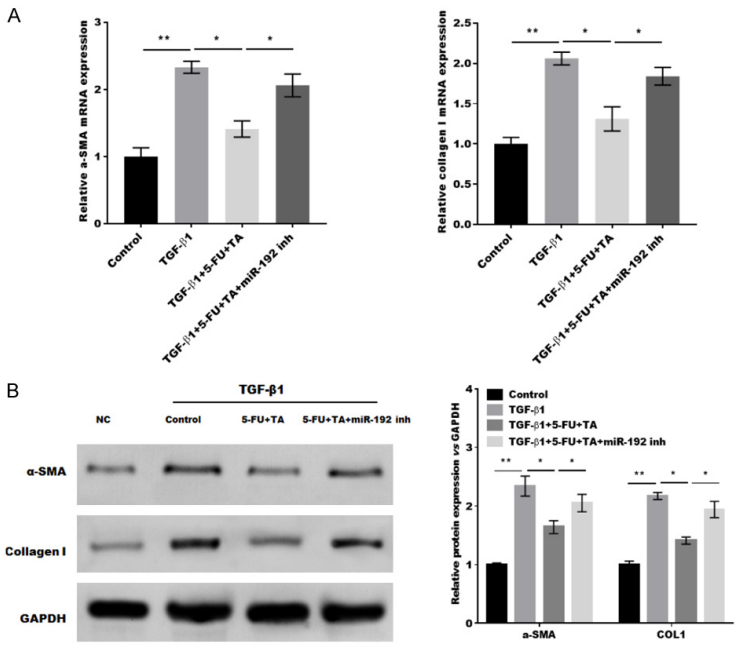


Figure 4. Selective inhibition of miR-192 blocked the improvement of TA combined with 5-FU on urethral fibrosis. A. RT-PCR analysis of α-SMA and collagen I mRNA expression in primary urethral scar fibroblasts isolated from urethral scar tissues and induced by TGF-β1 (10 ng/ml for 24 h), 5-FU/TA in the absence or presence of miR-192 inhibitor. B. Western blot analysis for α-SMA and collagen I protein level in primary urethral scar fibroblasts isolated from urethral scar tissues and induced by TGF-β1 (10 ng/ml for 24 h), 5-FU/TA in the absence or presence of miR-192 inhibitor. RT-PCR, Reverse Transcription-Polymerase Chain Reaction. 5-FU/TA, TA combined with 5-FU treatment. **P*<0.05, ***P*<0.01.

in the absence or presence of ATG7. miR-192-5p obviously decreased the expression of ATG7 and cell fibrosis, but these effects were blocked after ATG7 overexpression (**Figure 6B**).

Overexpression of miR-192-5p in rats inhibit urethral autophagy and fibrosis

To explore the role of miR-192-5p in urethral scar progression *in vivo*, urethral scar rat model was established according to previously studied. The model rats were injected with or without agomir-192-5p. Next, the mRNA expression of α-SMA and collagen I were assessed in urethral scar tissue. As shown in **Figure 7A**, the mRNA level of α-SMA and collagen I were dramatically increased in urethral tissues of animal model compared to the control group and decreased after miR-192-5p overexpression.

To further verify the implications of the miR-192-5p/ATG7 mediate autophagy in urethral scar development. Immunohistochemistry (IHC) staining was used to detect the fibrosis and autophagy changes of urethral scar tissue in these rats. Compared with the control rat, the urethral scar rat model obviously increased the fibrosis marker α-SMA and the autophagy marker LC3B, while the infection with miR-192-5p improved urethral fibrosis and autophagy in the urethral scar model rats (**Figure 7B**). Finally, we measured the expression of α-SMA, collagen I, ATG7 and LC3B in the urethral scar tissues of model rats by Western blot analysis (**Figure 7C**). Results showed that, compared with normal control rats, the expression of α-SMA, collagen I, ATG7 and LC3B in the urethral scar rats were significantly elevated, while the expression of α-SMA, collagen I, ATG7 and LC3B in the urethral scar rats was notably reduced by infection with miR-192-5p.

Discussion

Urethral stricture is a stricture of the urethra caused by scar tissue [18]. It can lead to obstructive voiding dysfunction and may cause long-term damage to the entire urinary tract [19]. Here, we investigated the effect of combined use of 5-FU and TA in improving urethral stricture. The current study demonstrated that miR-192 mediated the improvement of urethral scar by triamcinolone acetonide combined with 5-fluorouracil through inhibiting autophagy. The following findings were demonstrated by our results: (1) TA combined with 5-FU suppresses urethral fibrosis in vivo and in vitro; (2) miR-192 is involved in the function of TA combined with 5-FU suppresses urethral fibrosis; (3) Selective inhibition of miR-192 blocked the improvement of TA combined with 5-FU on urethral fibrosis; (4) miR-192 inhibits autophagy by directly targeting ATG7; (5) Overexpression of miR-192 in rats inhibit urethral autophagy and fibrosis. These data reveal that miR-192 could be a potential therapy target for urethral stricture.

In the therapy of keloids and hypertrophic scars, glucocorticoid injection is still the first-line treatment, but the treatment of glucocorti-

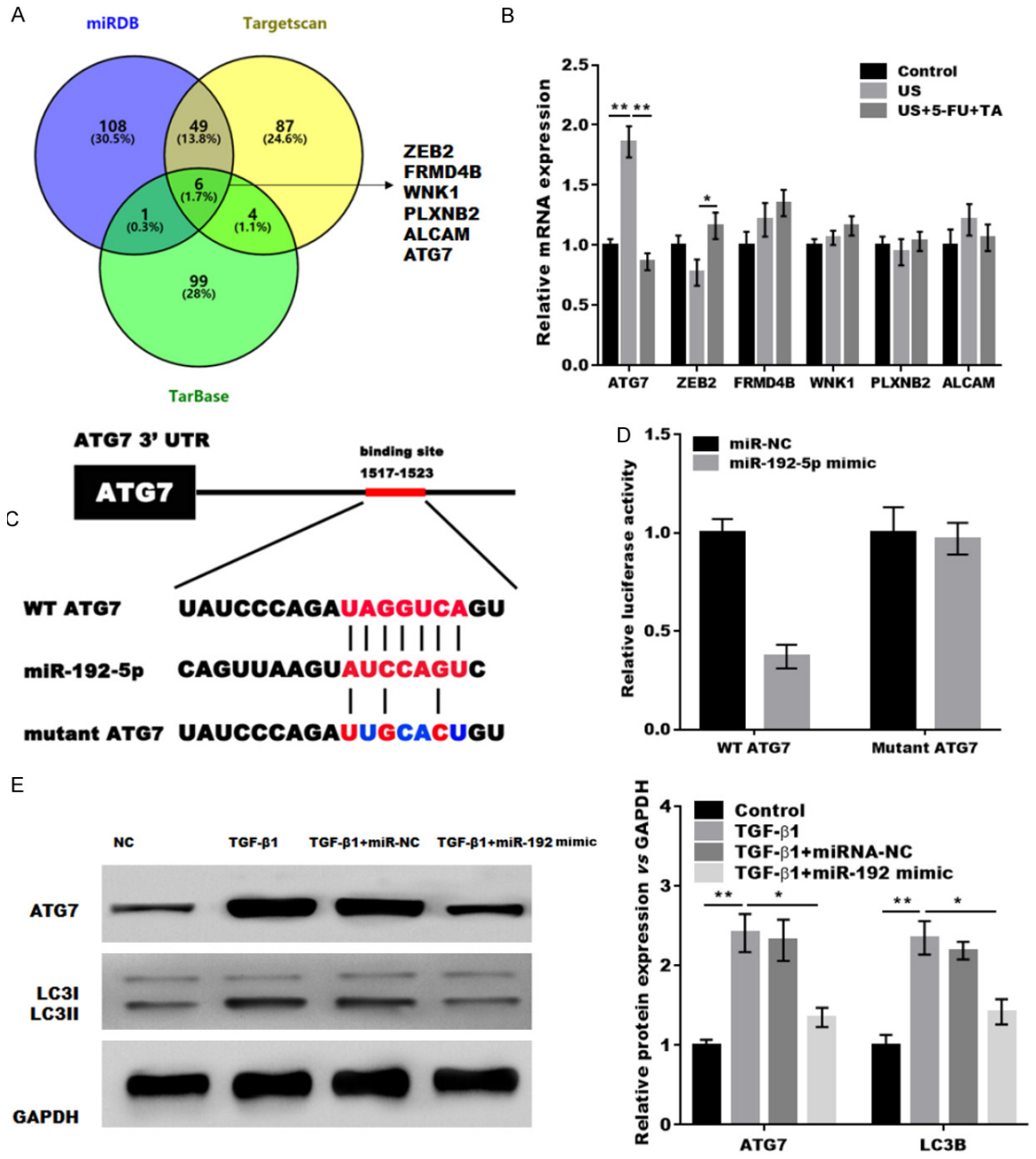


Figure 5. miR-192-5p inhibits autophagy by directly targeting ATG7. A and B. Venn diagram of the miR-192-5p target, predicted by targetscan, miRDB and TarBase. B. The mRNA expression of six target genes (ATG7, ZEB2, FRMD4B, WNK1, PLXNB2 and ALCAM) in rat urethral tissues of urethral scar rats treated with or without 5-FU/TA were detected by RT-PCR array. C. Schematic representation of the miRNA-192-5p site in ATG7-3'UTR. D. Luciferase activity was detected in fibroblasts co-transfected with miRNA-192-5p mimic and luciferase reporters containing wild type or mutant ATG7-3'UTR. E. The protein expression of ATG7 and LC3B was detected by western blot in urethral scar fibroblasts induced by TGF-β1 (10 ng/ml for 24 h) with or without miR-192-5p mimic. RT-PCR, Reverse Transcription-Polymerase Chain Reaction. *P<0.05, **P<0.01.

coid has many side effects when it been used alone, which limits its clinical application [20]. Recently years, lots of study shown that TA combined with 5-FU has achieved good clinical results [21]. Sunil Srivastava et al. documented that TA, 5-FU and their combination are all effective to the keloid scars, the combination of TA and 5-FU increases the drug efficacy and

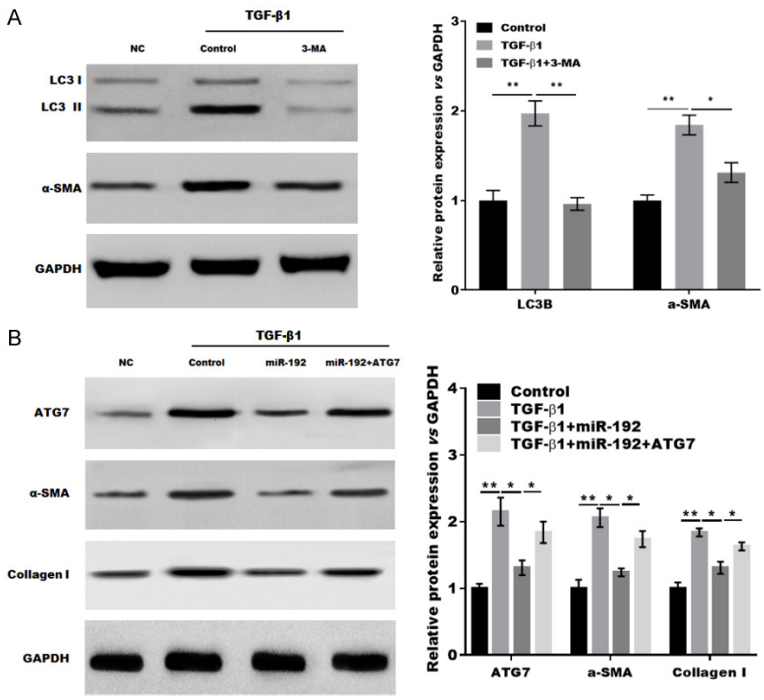
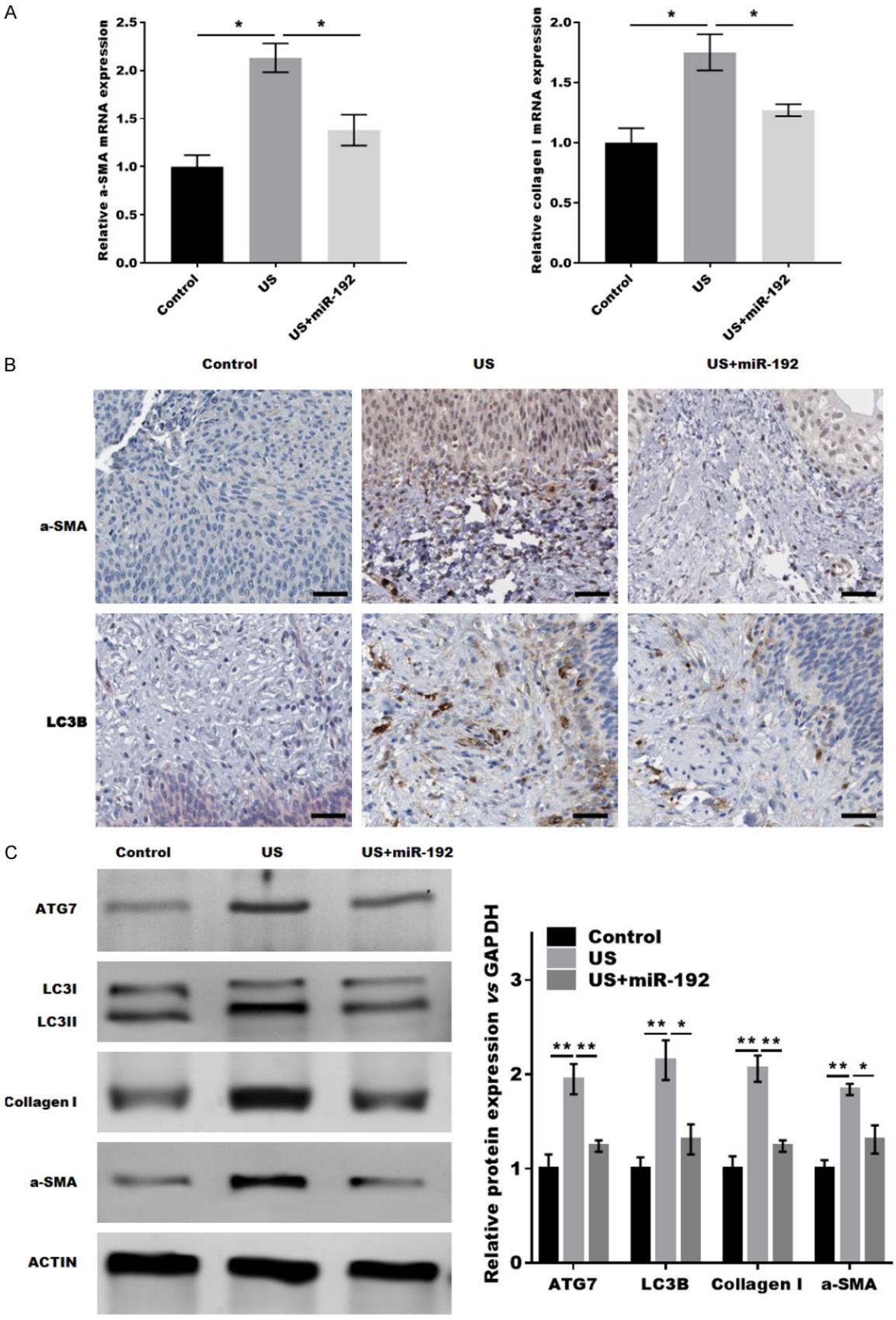


Figure 6. ATG7 mediates the inhibition of urethral fibrosis by miR-192-5p. A. Western blot analysis for LC3B and α-SMA protein level in urethral fibrosis model induced by TGF-β1 (10 ng/ml for 24 h) with or without 3-MA (10 mM). B. Western blot analysis for ATG7, α-SMA and collagen I in urethral scar fibroblasts induced by TGF-β1 (10 ng/ml for 24 h) transfected with miR-192-5p with or without ATG7. 3-MA, 3-Methyladenine. **P*<0.05, ***P*<0.01.

reduces the toxic side effects of drugs compared with single drugs [22]. YiMing Ren et al. suggested the combination of TA and 5-FU is more suitable for the prevention and treatment of keloids and hypertrophic scars and it improves the scar height and patient satisfaction, and has less side effects [23]. TA also been used in the therapy of urethral stricture, Kamyar TT et al. documented that TA injection after internal urethrotomy can delay the recurrence of urethral stricture [6]. Santosh Kumar et al. clarified that holmium laser urethrotomy combined with intralesional injection of triamcinolone is a safe and effective minimally invasive therapy for urethral strictures [24]. However, the study of triamcinolone acetonide combined with 5-FU in the treatment of urethral stricture is rare. In the current study, we found that the combination of 5-FU and TA can inhibit the urethral fibrosis in vivo an in vitro. However, the underlying mechanisms are still unclear.

The mechanisms of 5-FU combined with TA in the therapy of hypertrophic scars and keloids is complicated. TA inhibits the proliferation of scar fibroblast, enhance the degradation of collagen [25]. 5-FU inhibits the synthesis of thymidylate synthase, while thymidylate synthase inhibits DNA synthesis [26]. In addition, 5-FU blocked the expression of the Col1A2 gene, this result indicate that 5-FU can inhibit the growth of type I collagen fibers and fibroblasts [27]. 5-FU combined with TA induced the G2 cell cycle arrest and the downregulation of VEGF and the inhibition on Col-1 and MMP-2 synthesis in keloid fibroblasts [28]. However, the upstream regulatory mechanisms underlying 5-FU combined with TA inhibition of collagen synthesis and the cell fibrosis still unclear.

It is well accepted that microRNAs play a crucial role in the development of fibrosis in many organs, including liver, kidney, and lung [29]. Therefore, we then investigated whether miRNA involve in the combination of 5-FU and TA in the therapy of hypertrophic scars and keloids. Kaile Zhang et al., shown that 26 miRNAs were abnormally expressed in the urethral scar tissues compared to the normal tissue by miRNA sequencing and RT-PCR [16]. Among these miRNAs, we found that the miR-192-5p was obviously decreased in urethral scar tissue of rat model compared with control. While, the combination of 5-FU and TA partly reversed the expression of miR-192-5p. More important, in vitro cell results also shown that the expressions of collagen I and α-SMA were up-regulated by TGF-β, however these effects were blocked by 5-FU and TA, while inhibition of miR-192-5p partly reversed the expression of collagen I and α-SMA. Our result indicates that miR-192-5p is participated in the inhibitory function of TA combined with 5-FU on urethral fibrosis. Next, the target of miR-192-5p was predicted by bioinformatics, and six common targets were obtained: ATG7, ZEB2, FRMD4B, WNK1, PLXNB2 and ALCAM. Among these six targets, only ATG7 was confirmed to mediate miR-


A
Relative a-SMA mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
US
US+miR-192
Relative collagen I mRNA expression
2.0
1.5
1.0
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Control
US
US+miR-192
B
Control
US
US+miR-192
a-SMA
LC3B
C
Control
US
US+miR-192
ATG7
LC3I
LC3II
Collagen I
a-SMA
ACTIN
Relative protein expression vs GAPDH
Control
US
US+miR-192
3
2
1
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ATG7
LC3B
Collagen I
a-SMA

Figure 7. Overexpression of miR-192-5p in rats inhibit urethral autophagy and fibrosis. A. RT-PCR analysis of α-SMA and collagen I mRNA expression in urethral tissues of urethral scar rats transfected with or without miR-192-5p. B. The expression of α-SMA and LC3B was detected by IHC in urethral tissues of urethral scar rats transfected with or without miR-192-5p. C. Western blot analysis for ATG7, LC3B, α-SMA and collagen I in urethral tissues of urethral scar rats transfected with or without miR-192-5p. IHC, Immunohistochemistry. *P<0.05, **P<0.01.

192-5p-regulated urethral fibrosis in vivo and in vitro. This result has also been confirmed in airway fibrosis, Lili Lou et al., document miRNA-192-5p may attenuate airway remodeling and autophagy in asthma via targeting MMP-16 and ATG7 [30]. ATG7 is contribute to two ubiquitin-like protein conjugation systems, the Atg8 conjugation system and Atg12 conjugation system, as one of components during autophagy [31]. ATG7 play an important role in the initiation of autophagosome formation. Based on the above data and literature, we come to a conclusion that the combination of 5-FU and TA inhibits urethral fibrosis by suppressing autophagy. This is consistent with other studies in other diseases [32-34].

In conclusion, this study has shown that TA combined with 5-FU suppresses urethral fibrosis and miR-192-5p levels are decreased in urethral scar tissue. The protect effect of TA combined with 5-FU on urethral fibrosis was block by miR-192-5p inhibition. MiR-192-5p mediated the improvement of urethral scar by triamcinolone acetonide combined with 5-fluorouracil by targeting ATG7 and inhibiting autophagy. Therefore, we documented that TA combined with 5-FU suppresses urethral scar fibroblasts autophagy and fibrosis by increasing miR-192-5p expression.

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Disclosure of conflict of interest

None.

Abbreviations

5-FU, 5-fluorouracil; TAC, triamcinolone acetonide; RT-PCR, Reverse transcription PCR; ATG7, autophagy related gene 7; SD rat, Sprague Dawley rat; DMEM, Dulbecco's modification of Eagle's medium Dulbecco; COL1, Collagen I; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinyl difluoride; 3'-UTR, 3'untranslated region; SD, standard deviation; ANOVA, Analysis of Variance; DAB, 3,3'-diaminobenzidine; BCA, bicinchoninic acid; α-SMA, alpha-smooth muscle aorta; TGF-β1, transforming growth factor-β 1; miRNA, microRNA; PBS, phosphate buffer saline; FBS, fetal bovine serum; NC, normal control; SPSS, Statistical Product and Service Solutions.

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References

[1] King C and Rourke KF. Urethral stricture is frequently a morbid condition: incidence and factors associated with complications related to urethral stricture. Urology 2019; 132: 189-194.

[2] Strittmatter F, Beck V, Stief CG and Tritschler S. Urethral stricture: from diagnostics to appropriate treatment. Urologe A 2017; 56: 1047-1057.

[3] Stack RS and Schlossberg SM. General principles in treatment of urethral strictures. Urologe A 1998; 37: 10-20.

[4] Buckley JC, Heyns C, Gilling P and Carney J. SIU/ICUD consultation on urethral strictures: dilation, internal urethrotomy, and stenting of male anterior urethral strictures. Urology 2014; 83: S18-22.

[5] Ayyildiz A, Nuhoglu B, Gulerkaya B, Caydere M, Ustun H, Germiyanoglu C and Erol D. Effect of intraurethral Mitomycin-C on healing and fibrosis in rats with experimentally induced urethral stricture. Int J Urol 2004; 11: 1122-1126.

[6] Tavakkoli Tabassi K, Yarmohamadi A and Mohammadi S. Triamcinolone injection following internal urethrotomy for treatment of urethral stricture. Urol J 2011; 8: 132-136.

[7] Jaidane M, Ali-El-Dein B, Ounaies A, Hafez AT, Mohsen T and Bazeed M. The use of halofuginone in limiting urethral stricture formation and recurrence: an experimental study in rabbits. J Urol 2003; 170: 2049-2052.

[8] Ali L, Shahzad M, Orakzai N, Khan I and Ahmad M. Efficacy of mitomycin C in reducing recurrence of anterior urethral stricture after inter-

nal optical urethrotomy. Korean J Urol 2015; 56: 650-655.
[9] Kurt O, Gevher F, Yazici CM, Erboga M, Dogru M and Aktas C. Effect of mitomycin - C and triamcinolone on preventing urethral strictures. Int Braz J Urol 2017; 43: 939-945.
[10] Yang SY, Yang JY, Hsiao YC and Chuang SS. A comparison of gene expression of decorin and MMP13 in hypertrophic scars treated with calcium channel blocker, steroid, and interferon: a human-scar-carrying animal model study. Dermatol Surg 2017; 43 Suppl 1: S37-S46.
[11] Morelli Coppola M, Salzillo R, Segreto F and Persichetti P. Triamcinolone acetonide intralesional injection for the treatment of keloid scars: patient selection and perspectives. Clin Cosmet Investig Dermatol 2018; 11: 387-396.
[12] Diegelmann RF, Bryant CP and Cohen IK. Tissue alpha-globulins in keloid formation. Plast Reconstr Surg 1977; 59: 418-423.
[13] Soliman NA, Abd-Ellatif RN, ELSaadany AA, Shalaby SM and Bedeer AE. Luteolin and 5-flurouracil act synergistically to induce cellular weapons in experimentally induced Solid Ehrlich Carcinoma: realistic role of P53; a guardian fights in a cellular battle. Chem Biol Interact 2019; 310: 108740.
[14] Saha AK and Mukhopadhyay M. A comparative clinical study on role of 5-flurouracil versus triamcinolone in the treatment of keloids. Indian J Surg 2012; 74: 326-329.
[15] Davison SP, Dayan JH, Clemens MW, Sonni S, Wang A and Crane A. Efficacy of intralesional 5-fluorouracil and triamcinolone in the treatment of keloids. Aesthet Surg J 2009; 29: 40-46.
[16] Zhang K, Chen J, Zhang D, Wang L, Zhao W, Lin DY, Chen R, Xie H, Hu X, Fang X and Fu Q. microRNA expression profiles of scar and normal tissue from patients with posterior urethral stricture caused by pelvic fracture urethral distraction defects. Int J Mol Med 2018; 41: 2733-2743.
[17] An G, Liang S, Sheng C, Liu Y and Yao W. Upregulation of microRNA-205 suppresses vascular endothelial growth factor expression-mediated PI3K/Akt signaling transduction in human keloid fibroblasts. Exp Biol Med (Maywood) 2017; 242: 275-285.
[18] Zaid UB, Lavien G and Peterson AC. Management of the recurrent male urethral stricture. Curr Urol Rep 2016; 17: 33.
[19] Smith TG 3rd. Current management of urethral stricture disease. Indian J Urol 2016; 32: 27-33.
[20] Yildirim ME, Kaynar M, Ozyuvali E, Badem H, Cakmak M, Kosem B and Cimentepe E. The effectiveness of local steroid injection after internal urethrotomy to avoid recurrence. Arch Ital Urol Androl 2016; 87: 295-298.
[21] Hietanen KE, Jarvinen TA, Huhtala H, Tolonen TT, Kuokkanen HO and Kaartinen IS. Treatment of keloid scars with intralesional triamcinolone and 5-fluorouracil injections - a randomized controlled trial. J Plast Reconstr Aesthet Surg 2019; 72: 4-11.
[22] Srivastava S, Patil A, Prakash C and Kumari H. Comparison of intralesional triamcinolone acetonide, 5-fluorouracil, and their combination in treatment of keloids. World J Plast Surg 2018; 7: 212-219.
[23] Ren Y, Zhou X, Wei Z, Lin W, Fan B and Feng S. Efficacy and safety of triamcinolone acetonide alone and in combination with 5-fluorouracil for treating hypertrophic scars and keloids: a systematic review and meta-analysis. Int Wound J 2017; 14: 480-487.
[24] Kumar S, Kapoor A, Ganesamoni R, Nanjappa B, Sharma V and Mete UK. Efficacy of holmium laser urethrotomy in combination with intralesional triamcinolone in the treatment of anterior urethral stricture. Korean J Urol 2012; 53: 614-618.
[25] Carroll LA, Hanasono MM, Mikulec AA, Kita M and Koch RJ. Triamcinolone stimulates bFGF production and inhibits TGF-beta1 production by human dermal fibroblasts. Dermatol Surg 2002; 28: 704-709.
[26] Grogan BC, Parker JB, Guminski AF and Stivers JT. Effect of the thymidylate synthase inhibitors on dUTP and TTP pool levels and the activities of DNA repair glycosylases on uracil and 5-fluorouracil in DNA. Biochemistry 2011; 50: 618-627.
[27] Wendling J, Marchand A, Mauviel A and Verrecchia F. 5-fluorouracil blocks transforming growth factor-beta-induced alpha 2 type I collagen gene (COL1A2) expression in human fibroblasts via c-Jun NH2-terminal kinase/activator protein-1 activation. Mol Pharmacol 2003; 64: 707-713.
[28] Huang L, Wong YP, Cai YJ, Lung I, Leung CS and Burd A. Low-dose 5-fluorouracil induces cell cycle G2 arrest and apoptosis in keloid fibroblasts. Br J Dermatol 2010; 163: 1181-1185.
[29] Henry TW, Mendoza FA and Jimenez SA. Role of microRNA in the pathogenesis of systemic sclerosis tissue fibrosis and vasculopathy. Autoimmun Rev 2019; 18: 102396.
[30] Lou L, Tian M, Chang J, Li F and Zhang G. MiRNA-192-5p attenuates airway remodeling and autophagy in asthma by targeting MMP-16 and ATG7. Biomed Pharmacother 2020; 122: 109692.
[31] Arakawa S, Honda S, Yamaguchi H and Shimizu S. Molecular mechanisms and physiological roles of Atg5/Atg7-independent alternative autophagy. Proc Jpn Acad Ser B Phys Biol Sci 2017; 93: 378-385.

[32] Wu J, Xing C, Zhang L, Mao H, Chen X, Liang M, Wang F, Ren H, Cui H, Jiang A, Wang Z, Zou M and Ji Y. Autophagy promotes fibrosis and apoptosis in the peritoneum during long-term peritoneal dialysis. J Cell Mol Med 2018; 22: 1190-1201.

[33] Chen J, Yu Y, Li S, Liu Y, Zhou S, Cao S, Yin J and Li G. MicroRNA-30a ameliorates hepatic fibrosis by inhibiting Beclin1-mediated autophagy. J Cell Mol Med 2017; 21: 3679-3692.

[34] Meng D, Li Z, Wang G, Ling L, Wu Y and Zhang C. Carvedilol attenuates liver fibrosis by suppressing autophagy and promoting apoptosis in hepatic stellate cells. Biomed Pharmacother 2018; 108: 1617-1627.