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MicroRNA let-7i induced autophagy to protect T cell from apoptosis by targeting IGF1R

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ABSTRACT

MicroRNA let-7i is up-regulated in T cells from patients with Ankylosing Spondylitis (AS). In this study, we investigated the role of let-7i in T cells survival. Our results demonstrated down-regulation of insulin-like growth factor-1 receptor (IGF1R) in T cells from patients with AS. Luciferase reporter assay suggested IGF1R as direct target of let-7i. Overexpression of let-7i in Jurkat cells significantly suppressed IGF1R expression, which mimicked the action of IGF1R siRNA. IGF1R inhibition led to a strinking decrease in phosphorylation of mTOR and Akt, down-regulation of Bcl-2, up-regulation of Bax and cleavage of caspase 3 and PARP. Meanwhile, IGF1R inhibition induced autophagy. Autophagy induced by let-7i overexpression contributed to protect cells from apoptosis. Our data indicated that let-7i might control T cells fates in AS by targeting IGF1R.

1. Introduction

Ankylosing Spondylitis (AS) is a chronic inflammatory disease that characterizes as pain, stiffness, and inflammation of the joints between the spinal bones. Although genome-wide association studies (GWAS) have explored numbers of genes associated with AS [1], the pathogenesis of AS remains unclear. It has been recognized that HLA-B27 is the most important risk for AS [2]. Bacterial infection has been proposed to be implicated in pathological process of AS, which is construed as mimicry between the HLA-B27 antigens and the bacterial cells [3]. Infection also stimulates the up-regulation of T cells to maintain immune homeostasis [4]. There are increasing findings suggesting the involvement of T cells in AS [5,6]. Recent studies reveal that disordered T cells are observed in peripheral blood and inflammatory joints from patients with AS [7,8]. However, the precise mechanism of how T cells contribute to AS remains unknown.

MicroRNAs (miRNA) are a large family of non-coding small RNAs that are composed of 18–25 nucleotides. MiRNAs inhibit the expression of multiple target genes by binding to their 3′UTR. MiRNAs have been implicated in inflammatory and autoimmune diseases, including rheumatoid arthritis [9], and multiple sclerosis [10]. Amounts of investigations have confirmed the key role of miR-146a in immune function [11]. Furthermore, miR-155 is critical for inflammation and T regulatory cell function [12]. Let-7 miRNA family is composed of 12 members controlling innate immune response [13]. For example, inhibition of let-7a decreases production of allergic cytokines and alleviates the phenotype of asthma [14]. Upregulation of let-7i in T cells from AS has been found, which contributes to IFN-γ production [15]. All the data suggest the important inflammatory role of miRNA.

Based on Targetscan analysis, insulin-like growth factor-1 receptor (IGF1R) was predicted to be a target of let-7i. IGF1R is located on T cell surface determining activation of T cells [16]. The increasing evidences have demonstrated down-regulation of IGF1R at the early stage of T cells activation followed by increasing IGF1R level along with T cells activation [17,18]. High IGF1R level is necessary to maintain T cells activity [19]. Considering up-regulation of let-7i in T cells from AS and the key role of IGF1R in T cells activation, we hypothesized that let-7i is involved in AS by targeting IGF1R.

In this study, we confirmed that IGF1R was down-regulated in T cells from AS. It was further demonstrated that IGF1R served as the target of let-7i and was inhibited by let-7i overexpression in the human leukemic T cell line (Jurkat). It was also evidenced that IGF1R inhibition by either let-7i or siRNA influenced its downstream genes, including mTOR, Akt, Bcl-2, Bax, cleavage of caspase 3 and PARP, and simultaneously caused autophagy. Interestingly, Autophagy induced by let-7i overexpression was conducive to pro-

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tect T cells from apoptosis. Our results might illustrate the novel roles of let-7i and IGF1R in AS.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies against IGF1R, Akt, phosphor-Akt (Ser473), LC3B, and mTOR were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies against Bcl-2, Bax, caspase 3 and PARP were obtained from Abcam (Cambridge, MA). The antibody to beta-actin (pAb) was from Anbo Biotechnology Company (San Francisco, CA, USA). RIPA lysis buffer was purchased from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Carlsbad, CA). 3-Methyladenine (3-MA) was obtained from Sigma Chemical Co. (USA).

2.2. Study population

Thirty-one HLA-B27-positive patients were recruited as case subjects from the Shandong provincial Hospital affiliated to Shandong University, all of which were diagnosed with AS according to the modified New York criteria [20]. Twenty-six age- and sex-matched healthy volunteers, who had no autoimmune diseases and were HLA-B27-negative, were recruited randomly as control subjects from Shandong province and the surrounding area. Blood samples were collected at baseline after the last dose of immunosuppressants. This study was approved by the Medical Ethics Committee of Shandong University, and written informed consent was obtained from every participant.

2.3. Isolation of T cells

Venous bloods from AS patients and healthy volunteers were collected into tubes containing EDTA, from which peripheral blood mononuclear cells (PBMC) were isolation by Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA) at 300*g* and 4 °C for 10 min. T cells were further purified using magnetic beads coated with anti-human CD3 (Miltenyi Biotec, GmbH, Gladbach, Germany). The T cells were incubated at a density of 1×10^6/ml in RPMI-1640 medium (Hyclone Inc, USA) supplemented with 10% fetal bovine serum (Hyclone, Australian), 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in 5% CO_2.

2.4. Cell culture

The human Jurkat T cell line (ATCC, Manassas, VA) was cultured in RPMI 1640 medium (Hyclone Inc., USA) supplemented with 10% fetal bovine serum (Hyclone, Austrilian), 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in 5% CO_2.

2.5. Quantitative RT-PCR

The total RNA was extracted from the purified T cells with the RNeasy mini kit (Qiagen, Gaithersburg, MD, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The synthesized cDNA was used as template to amplify IGF1R with the specific primers (Forward 5′ CCG CTGCCAGAAAATGTGCCCA 3′ and Reverse 5′ TGTCGTTGTCAGGC GCGCTG 3′) [21], and GADPH with the primers (Forward 5′-GGT GAAGGTCGGAGTCAACGG-3′ and Reverse 5′-GGTCATGAGTC CTTCCACGATACC-3′) [22].

2.6. Luciferase reporter assay

The let-7i targeting sequence locating at the position 2619–2626 of IGF1R 3′UTR was amplified using forward (5′ TCTC GAGCCCCCAAACATTTATCTACCTCACT 3′) and reverse (5′ TCTCAA GCTTAGGTGCTGAGAAAGGTGAGATGTAT 3′) primers containing Sac I and Hind III restriction sites. The corresponding mutant was amplified using forward (5′ TCTCGAGCCCCCAAACATTTATCATGG AGCT 3′) and reverse (5′ TCTCAAGCTTAGGTGCTGAGAAAGGTGAG ATGTAT 3′) primers. The PCR products were digested and ligated into pHSA-MIR-REPORT (Ambion, Grand Island, NY, USA) to form pMIR-IGF1R-WT and pMIR-IGF1R-MT. The 293T (a human embryonic kidney cell line) was co-transfected with pMIR-IGF1R-WT or pMIR-IGF1R-MT and mature let-7i using Lipofectamine 2000 reagent (Invitrogen), and pRL-SV40 plasmid (Promega) was used as a normalizing control. After 24 h incubation, luciferase and renilla activities were determined with the Dual-Luciferase Assay (Promega) according to the manufacturer's instructions.

2.7. Transient transfection

Jurkat T cells were transfected with let-7i mimic or anti-let-7i using Lipofectamine 2000 reagent and cultured for 48 h. Then, the cells were collected and washed with ice-cold PBS for subsequent analysis. Jurkat cells were also transfected with small interfering RNA (siRNA) targeting IGF1R using Lipofectamine 2000 reagent, which were collected and rinsed with ice-cold PBS for subsequent analysis.

2.8. Western blot analysis

The whole-cell lysates were obtained using RIPA lysis buffer. The protein concentration was determined using the Bradford assay. Equal amounts of proteins were loaded onto 10% SDS–PAGE. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk for 1 h at room temperature followed by probed with the corresponding antibodies overnight at 4 °C. After washed three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The labeled proteins were detected using the enhanced chemiluminescence method and quantified using Alpha Imager2200.

2.9. Cell viability and LDH release assay

After 48 h post-transfection, the cells were incubated with MTT solution (0.5 mg/ml) at 37 °C for 4 h. Subsequently, the cells were washed with PBS, and the insoluble formazan product was dissolved in DMSO. The cell viability was determined by measuring the produced formazan at 490 nm using a SpectraMax M2. To determine LDH release, after transfection, supernatant was obtained by centrifugation at 400*g* for 5 min. Subsequently, 120 μl of supernatant from each well was transferred to a new a 96-well microplate and was mixed with 60 μl LDH working solution (Beyotime, Shanghai, China) followed by incubation for 30 min at room temperature. LDH activity was detected at 490 nm using a SpectraMax M2. The total cell lysate was set as 100%.

2.10. Immunofluorescence

The cells were collected and fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked with 10% normal goat serum for 1 h at room temperature. After washed three times with PBST, the cells were

stained with IGF1R or LC3 antibodies overnight at 4 °C followed by incubation with FITC-conjugated secondary antibody IgG for 2 h. After washed three times with PBST, the images of cells were captured using a fluorescence microscope (LEICA DMIRE2).

2.11. Flow cytometric analysis

The transfected cells were harvested by centrifugation at 1000*g* for 5 min. After washed with PBS three times, the cells were resuspended in binding buffer containing annexinV-FITC and propidiumiodide for 10 min in the dark at room temperature. Subsequently, the labeled cells were analyzed by a flow cytometer (Beckman Coulter, Chicago, USA) and the data were processed using WinMDI 2.9 software.

2.12. Statistical analysis

All data were representative of at least three independent experiments. Student's *t*-test was used to analyze the data between two groups. One-way ANOVA was used to analyze the data among multiple groups. For all analyses, probability values less than 0.05 were considered significant. Statistical analysis was performed using the SPSS software (SPSS 18.0).

3. Results and discussion

3.1. Differential expression of IGF1R in T cells from AS patients

Increasing evidences have demonstrated that IGF1R is significantly associated with immunity and inflammation [11,23]. Remarkably, IGF1R regulation implicated in immune responses to diverse diseases is protean. For instance, overrepresentation of T cells expressing IGF1R has been found in Graves' disease [24], but IGF1R levels trend downward in several aging and chronic diseases [25]. Activation of adult lymphocytes causes a decline in IGF1R expression [16,18,26] whereas activation of cord blood mononuclear cells (CBMC) leads to an increase in IGF1R expression, which was thought to be dependent on the activated status [27]. Based on these discrepant data, we investigated the expression of IGF1R in T cells from AS patients. As shown in Fig. 1, flow cytometry analysis data illustrated that 78.6% of isolated CD3+ T cells expressed IGF1R in healthy volunteers whereas only 41.5% in AS patients, which was consistent with the results from RT-PCR that IGF1R mRNA levels were lower in AS group than that in normal group. Western blot analysis further testified that IGF1R expression was decreased in AS group. These results suggested down-regulation of IGF1R in T cells from AS patients, which was accordant to previous results [18,28]. However, our findings were differed from that in another report suggesting the up-regulation of IGF1R after mitogenic activation [26]. Intriguingly, IGF1R expression was time-dependent and was affected by environmental factors and stimulated conditions [17,18,26]. Down-regulation of IGF1R was observed at short notice after T cells activation while IGF1R level was gradually elevated with the long-term activation. The increasing IGF1R protected T cells from apoptosis through the transduction of Phosphoinositide 3-kinase (PI3 K)/Akt and Mitogen-activated protein kinase (MAPK) pathways [17,26]. IGF1R anti-apoptotic effects have been well documented in various tumor cells, including gastrointestinal stromal tumors [29], prostate cancer [30] and lung cancer [31], suggesting that IGF1R is closely correlated with cells survival. However, a strinking decline in IGF1R observed in this study seemed to be contradictory to the fact

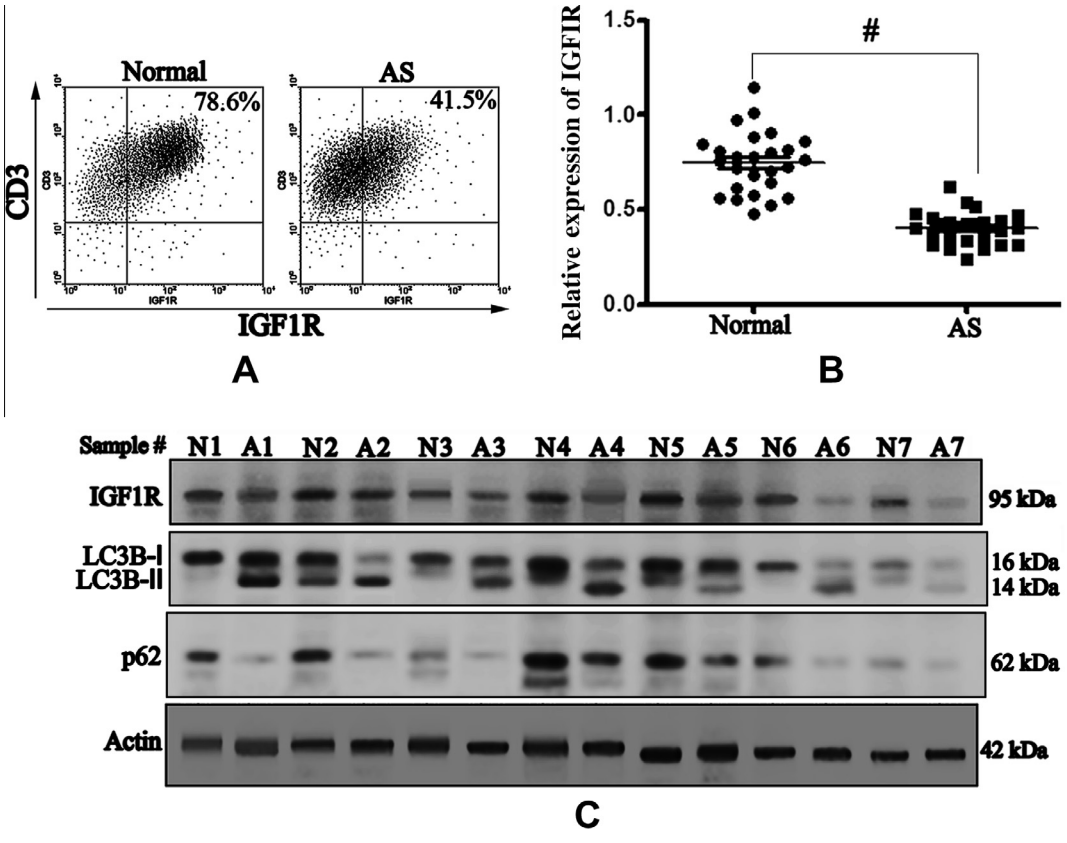

Fig. 1. Down-regulation of IGF1R was observed in T cells from patients with AS compared with those healthy donors. (A) Detection of IGF1R expression in T cells from patients with AS and healthy controls by flow cytometry analysis. (B) The relative expression of IGF1R in T cells from patients with AS and healthy controls determined by qRT-PCR. (C) Western blot analysis of IGF1R expression in T cells from 7 patients with AS and 7 healthy controls. $^{\#}p < 0.05$ vs. normal group.

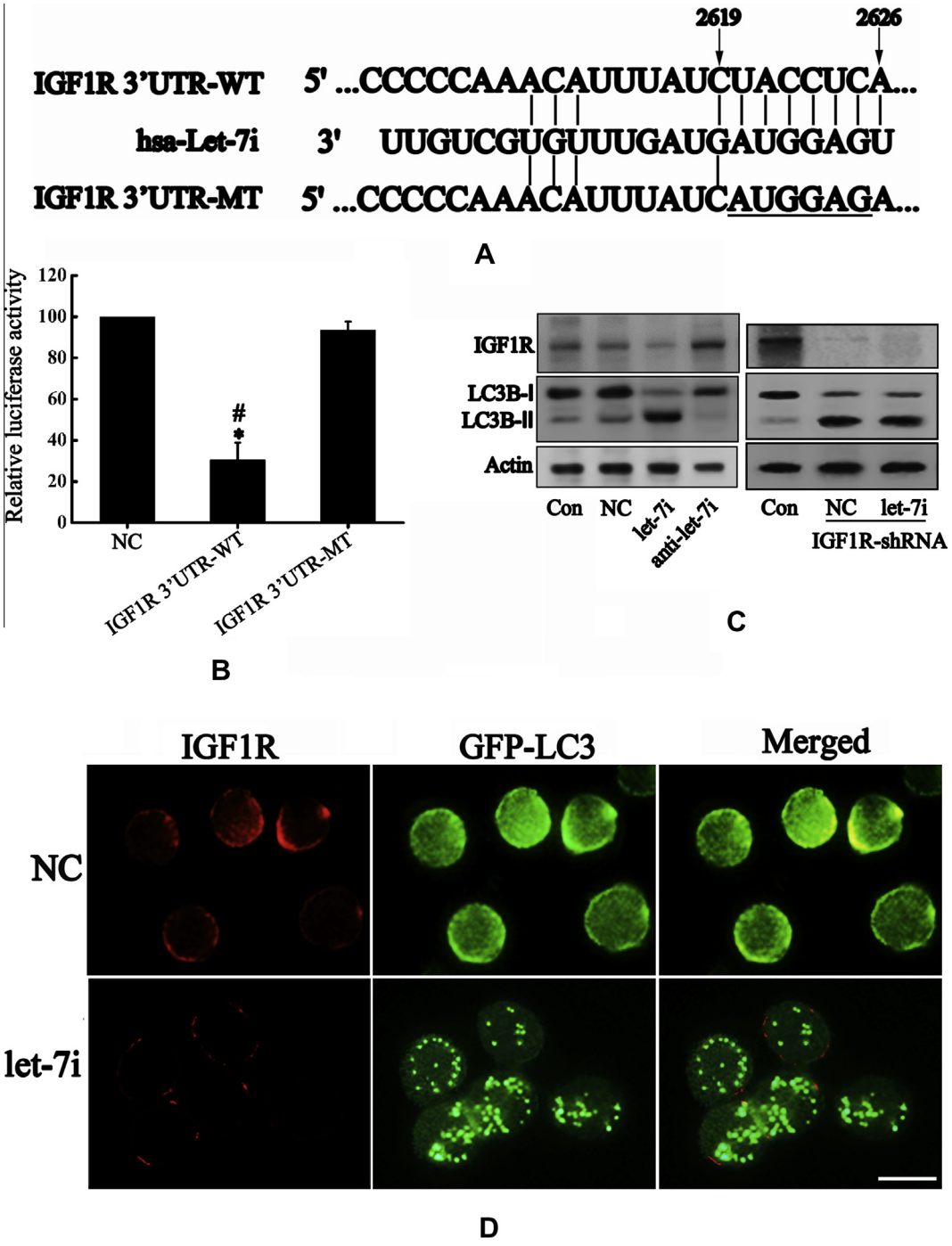

Fig. 2. Overexpression of let-7i induced autophagy by directly targeting IGF1R. (A) Diagram of IGF1R 3′UTR containing the predicted let-7ibinding site. (B) Luciferase reporter assay in 293T cells. (C) Western blot analysis of IGF1R expression and LC3-II accumulation. (D) Detection of IGF1R expression and formation of LC3 puncta by immunofluorescence. Scale bar, 10 μm. $^{*}p < 0.01$ vs. NC group; $^{\#}p < 0.01$ vs. mutant group.

that numbers of T cells were required for immune response in AS [32]. Therefore, it was necessary to investigate what reduced IGF1R and whether down-regulation of IGF1R influenced T cells survival. We also examined differential expression of LC3 and p62 in T cells from AS patients and healthy volunteers. The results suggested that LC3B-II, a marker of autophagy, was up-regulated in T cells from AS patients, which was responsible for the decrease in p62 level in AS patients (Fig. 1C). These data revealed that IGF1R might be implicated in autophagy.

3.2. Overexpression of let-7i induced autophagy by targeting IGF1R

Previous report has indicated that let-7i is up-regulated in T cells from AS [15]. The let-7 family members have been demonstrated to target directly IGF1R in head and neck cancer cells, prostate cancer cells, hepatocellular carcinoma cells, and spermatogonia [33–35]. Because we found down-regulation of IGF1R in T cells from AS, we further investigated whether let-7i targeted directly IGF1R. IGF1R 3′UTR containing targeting site or its

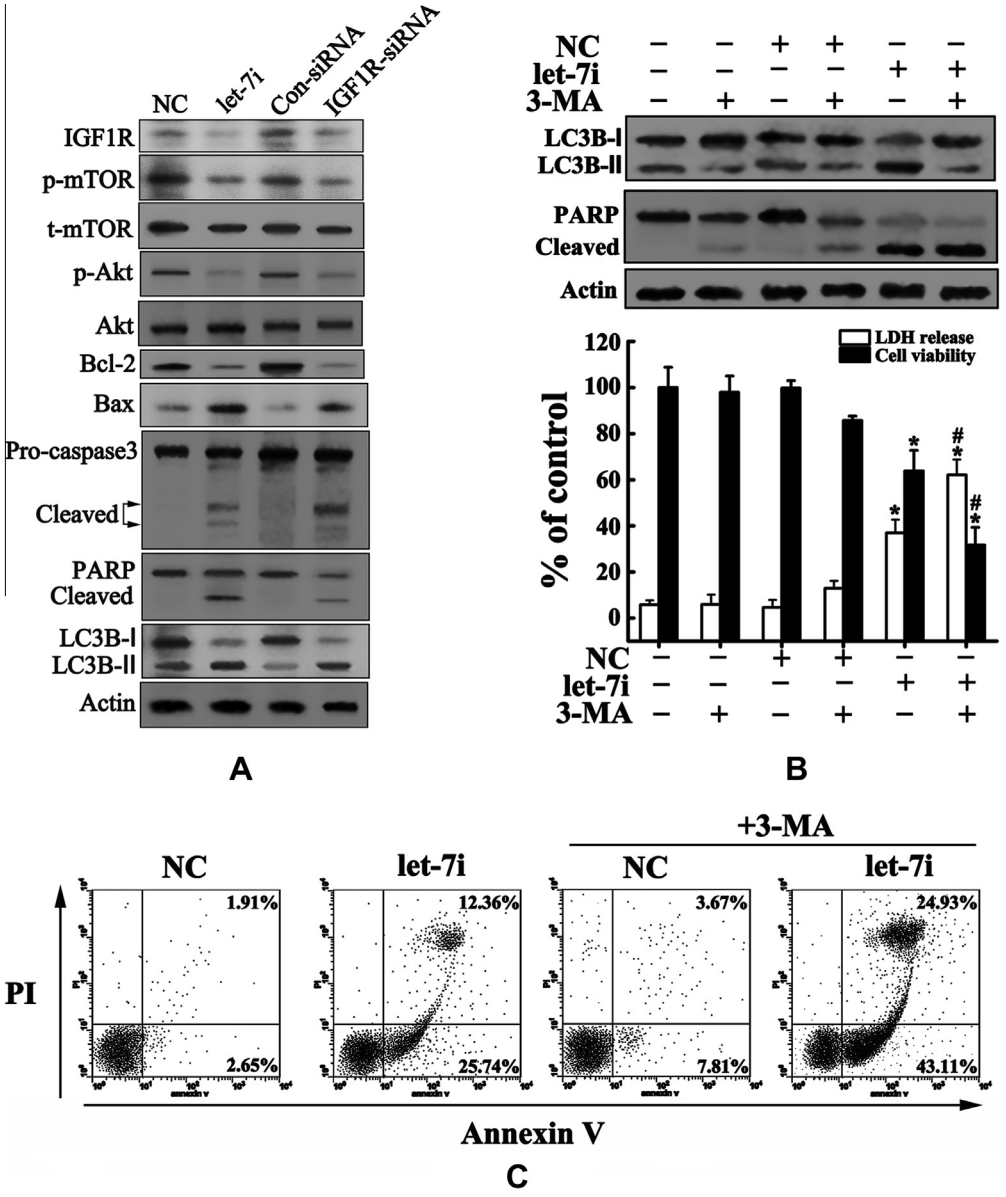

Fig. 3. Overexpression of let-7i induced autophagy to protect cells from apoptosis. (A) Western blot analysis of the proteins involved in apoptosis and autophagy. (B) Effect of autophagy inhibitor on cells viability, LDH release and PARP cleavage. (C) Effect of autophagy inhibitor on cells apoptosis determined by flow cytometry analysis. $^{*}p < 0.05$ vs. NC group; $^{\#}p < 0.05$ vs. the group of let-7i tranfection.

mutant were cloned into 3′UTR of the luciferase reporter gene (Fig. 2A). The results revealed that let-7i significantly suppressed luciferase activity co-transfecting with the vector carrying wide-type IGF1R 3′UTR whereas did not affect luciferase activity co-transfecting with the vector carrying mutant IGF1R 3′UTR (Fig. 2B). Consistently, overexpression of let-7i in Jurkat cells inhibited IGF1R expression while overexpression of anti-let-7i increased IGF1R level (Fig. 2C), suggesting that let-7i could participate in cellular events by targeting directly IGF1R. Intriguingly, let-7i transfection caused a dramatic increase in LC3B-II formation (Fig. 2C). In contrast, anti-let-7i transfection decreased LC3B-II accumulation (Fig. 2C). IGF1R knockdown by specific-shRNA also increased LC3B-II level compared with control shRNA-transfected cells, but let-7i overexpression in IGF1R knockdown cells did not significantly elevate LC3B-II level compared with IGF1R shRNA-transfected cells (Fig. 2C). Immunofluorescence analysis illustrated that let-7i transfection inhibited IGF1R expression and promoted the formation of LC3 puncta (Fig. 2D). These data indicated that overexpression of let-7i might lead to autophagy by targeting IGF1R. Several evidences have certified that IGF1R inhibition can induce autophagy [36,37]. Autophagy is an evolutionarily conserved manner that controlled degradation of damaged organelles in a cell. Autophagy has been proposed to be the hinge between cell survival and death. Under most cell stress, such as nutrient deprivation, autophagy plays a cytoprotective role. However, in response to severe or prolonged stress, autophagy can lead to cell death [38]. Autophagy can also act as an innate defence for infection or inflammation [39]. It has been observed that the levels of IL-1β and IL-18 are elevated in Atg16L1-deficient mice model of Crohn's disease [40]. Furthermore, autophagy-related protein, such as LC3II, ATG5 and ATG12, were found to be increased in the gut of patients with Ankylosing Spondylitis, which contributed to promote IL-23 expression [41]. All the above findings suggest that autophagy plays the critical role in mediating cells fates and organismal immune. Therefore, we hypothesized that let-7i transfection

induced autophagy to protect T cells from apoptosis by inhibiting IGF1R, which might facilitate immune defence in AS.

3.3. IGF1R Inhibition induced autophagy to rescue T cells

We further tested the effect of IGF1R inhibition on signal transduction in Jurkat cells. As shown in Fig. 3A, let-7i overexpression and IGF1R siRNA conducted the identical signaling consequences. Both let-7i overexpression and IGF1R siRNA significantly suppressed IGF1R expression. IGF1R inhibition blocked the phosphorylation of mTOR and Akt. IGF1R inhibition also reduced anti-apoptotic protein Bcl-2 and promoted the accumulation of Bax. All the results suggested that IGF1R inhibition accelerated cell apoptosis, which was confirmed by the increased cleavages of caspase 3 and PARP. IGF1R has been well-known for its participation in cell proliferation [29–31]. Consistent with the results in Fig. 2C, IGF1R inhibition led to accumulation of LC3B-II. Our results indicated that IGF1R inhibition might cause two signaling characteristics including autophagy and apoptosis. However, down-regulation of IGF1R was observed in T cells from AS patients, which was contradicted to the rising frequency of T cells in AS [32]. Therefore, autophagy induced by IGF1R inhibition likely played the key role in maintaining cell survival. To verify the relation between autophagy and apoptosis, we tested effect of 3-methyladenine (3-MA, an inhibitor of autophagy) on cell viability and cytotoxicity. The results showed that 3-MA reduced LC3B-II level, especially in the cells transfected with let-7i (Fig. 3B). Interestingly, the decrease in LC3B-II level was positively associated with the increase in PARP cleavage, suggesting that inhibition of autophagy further promoted cells apoptosis, which was also confirmed by MTT assay and LDH release assay (Fig. 3B). Although let-7i transfection led to about 35% reduce in cell viability compared with control group, attenuation of autophagy by 3-MA decreased approximately 70% of cell viability. Again, the results from flow cytometry analysis certified that let-7i overexpression induced apoptosis in about 25% of cells. However, 3-MA enlarged let-7i-induced apoptosis, which resulted in about 43% cells apoptosis (Fig. 3C). All the results suggested that IGF1R inhibition induced autophagy to prevent cell apoptosis.

In summary, we evidenced that IGF1R was down-regulated in T cells from the patients with AS. Combined with previous data that let-7i is up-regulated in T cells from the patients with AS, we tested whether IGF1R was the target of let-7i. The results that let-7i transfection inhibited luciferase activity and IGF1R expression suggested IGF1R as direct target of let-7i. IGF1R inhibition by let-7i overexpression induced autophagy to compete with cell apoptosis.

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