

## Original Article

# Autophagy-related genes affect drug resistance of mycobacteria by regulating autophagy

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**Abstract:** Objective: To investigate the effect of autophagy-related gene (ATG) on the drug resistance of mycobacteria by regulating autophagy. Methods: In the present study, primary macrophages were selected as objects of study. The cell lines with ATG13 and ATG6 interference and stable overexpression were constructed with Crisp/Cas9 technique and verified by fluorescence quantitative polymerase chain reaction (PCR) and western blotting, and the qualified cells were used for subsequent experiments. Then the above different mutant and wild-type cells were cultured in Dulbecco's Modified Eagle medium (DMEM) containing fetal bovine serum for 5 h, and Mycobacterium tuberculosis H37Rv was added, followed by co-culture for 4 h. The cells were treated and co-cultured with isoniazid (INH, 0.05 mg/L), rifampicin (RFP, 0.4 mg/L) and ethambutol (EMB, 25 mg/L) for 3 d. Then the cells were sampled and stained with monodansylcadaverine (MDC), and autophagy was observed. Finally, an appropriate number of cells were taken and cultured in the modified L-G medium, and the bacteria were counted. Results: The results of fluorescence quantitative PCR and western blotting revealed that the messenger ribonucleic acid (mRNA) transcription levels and protein expression levels of ATG13 and ATG6 in cells significantly declined after using Crisp/Cas9. The MDC staining showed that ATG13 and ATG6 interference could significantly reduce the number of autophagosomes in cells, while ATG13 and ATG6 overexpression could significantly increase the number of autophagosomes in cells. Compared with wild-type cells, the number of mycobacteria was obviously increased in mycobacterium-infected cells with ATG13 and ATG6 interference after they were treated with INH, RFP and EMB, displaying a significant difference ( $P < 0.05$ ), while the number of mycobacteria was obviously decreased in mycobacterium-infected cells with ATG13 and ATG6 overexpression after they were treated with INH, RFP and EMB, also a significant difference ( $P < 0.05$ ). Conclusion: ATG and other autophagy-related genes can affect the drug resistance of mycobacteria through regulating autophagy.

**Keywords:** ATG, autophagy, mycobacteria, drug resistance, MDC staining

## Introduction

Autophagy is an important means for the body's cells to resist the adverse external environment, which has become an important research field in apoptosis [1]. Autophagy refers to the process of degrading intracellular damaged organelles or foreign bodies using autophagosomes, lysosomes, and other autophagy-related organelles in the body under the control of autophagy-related genes (ATG) [2-4]. During this process, the formation of autophagosomes plays an important role in autophagy. In recent years, studies have found that [5-7] ATG plays a crucial regulatory role in the process of autophagy. It has also been found that ATG plays an important role in the initiation of autophagy and the fusion of autophagosomes with lyso-

somes. For example, ATG13 can be activated by adenosine monophosphate (AMP) kinase to promote autophagy, and ATG6 can accelerate the degradation of foreign bodies through promoting fusion of autophagosomes with lysosomes [8]. In recent years, with environmental deterioration in China, especially haze and other heavy weather in the northern region, the morbidity rate of tuberculosis has been increasing year by year [9]. Mycobacteria cause tuberculosis, and the drug resistance of mycobacteria has increased gradually due to the long-term abuse of antibiotics. Autophagy is an important way for cells to autonomously remove extracellular foreign bodies. Therefore, it is of significance to explore the correlation between autophagy and drug resistance of mycobacteria, so as to provide a theoretical and experimental

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**Table 1.** ATG13 and ATG6 primers

| Primer  | Primer sequence          |
|---------|--------------------------|
| ATG13-F | TGCTGATGCTGATCGTAGCTAG   |
| ATG13-R | GCTGATCGTAGCTAGCAGCTG    |
| ATG6-F  | GGCTGATCGAATCGTAGCTACGC  |
| ATG6-R  | TAGCTAGCTAACGTCGACTGACTC |

**Table 2.** ATG13 and ATG6 recognition target sequences

| Name      | Sequence                   |
|-----------|----------------------------|
| ATG13-1-F | TGCTGATCGTACGTACGCGTACG    |
| ATG13-1-R | CGTAGCTGTAGCTAGCTACGATCA   |
| ATG13-2-F | AGTAGCTAGCTAGGATCGATACAC   |
| ATG13-2-R | CGATGCTAGCTGCTAGCTAGATGC   |
| ATG6-1-F  | GGTAGCTAGGATCGATACGTACGTAC |
| ATG6-1-R  | CGTAGCTAGTTAGCTAGCTAGTAGC  |
| ATG6-2-F  | GTCGTAGCTGATTAGCTAGCTAGCT  |
| ATG6-2-R  | CGTAGCTAGCTGCTCGTAGCTAGC   |

basis for subsequent scientific research and clinical studies.

### General data

#### *Cell lines and bacterial strain source*

The primary macrophages used in the present study were preserved by our laboratory, and *Mycobacterium tuberculosis* was obtained from the National Institute for the Control of Pharmaceutical and Biological Products.

#### *Main reagents*

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum were purchased from Roche, 0.25% trypsin and EDTA reagents from Invitrogen, the lentiviral vector system from TAKARA, Crisp/Case9 kit from ABM, and the relevant recognition sites were predicted and designed by the online website (<https://chopchop.rc.fas.harvard.edu/index.php>). Isoniazide (INH), rifampicin (RFP), ethambutol (EMB), monodansylcadaverine (MDC) and modified L-G medium were bought from Sangon, and the ribonucleic acid (RNA) extraction kit, fluorescence quantitative polymerase chain reaction (PCR) and total protein extraction kit from TAKARA (Dalian, China).

*Construction of cell lines with ATG13 and ATG6 overexpression:* The cell lines with ATG13 and ATG6 overexpression were constructed in ac-

cordance with the procedure in the article of Wallis et al [10]. The ATG13 and ATG6 primers were synthesized by Sangon, and the primer sequences are shown in **Table 1**.

*Construction of mutants with ATG13 and ATG6 interference:* In the present study, the cell lines with ATG13 and ATG6 interference were constructed using Crisp/Case9 technique. First, the different targets in ATG13 and ATG6 were selected from the Crisp/Case9 online design website (<https://chopchop.rc.fas.harvard.edu/index.php>) and cleaved. The sgRNA sequences are shown in **Table 2** and 2 groups of recognition sites were selected per gene according to the instructions (ABM Crisp/Case9 kit, article No.: GMCA-001).

#### *Fluorescence quantitative PCR*

*RNA extraction:* RNA was extracted according to the instructions of the AXYGEN kit, as follows: (1) 0.1 g cryopreserved tissue specimens were taken from liquid nitrogen, dissolved on ice, added to 0.45 mL RNA Plus and ground into pieces in a pre-cooled mortar. Then the specimens were transferred into a 1.5 mL EP tube, added to 0.45 mL RNA Plus, washed and transferred into a centrifuge tube. (2) 200  $\mu$ L chloroform was added into the centrifuge tube, shaken vigorously for 15 s and placed on ice for 15 min, followed by (3) centrifugation at 12000 rpm and 4°C for 15 min. (4) The supernatant was transferred into the RNase-free EP tube, added to an equal amount of isopropanol, mixed evenly, and placed on ice for 10 min, followed by (5) centrifugation at 12000 rpm and 4°C for 10 min. (6) The supernatant was discarded, and 750  $\mu$ L 75% ethanol was added and mixed evenly, followed by centrifugation at 12000 rpm and 4°C for 10 min. (7) The supernatant was discarded, and the residual ethanol was removed as much as possible. (8) An appropriate amount of RNase-free water was added, and the mass of RNA extracted was determined, while the remaining RNA was used for reverse transcription [11].

#### *Fluorescence quantitative PCR*

In the present study, the fluorescence quantitative PCR kit was purchased from TAKARA, and the experiment was performed using the three-step method in accordance with the modified instructions [12]. The primers used are shown in **Table 3**.

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**Table 3.** Fluorescence quantitative PCR primers

| Primer   | Primer sequence           |
|----------|---------------------------|
| ATG13F-F | ATCGGCTAGCTAGCTACGATA     |
| ATG13F-R | TAGGCGGCATGCGAGGCTACGC    |
| ATG6F-F  | CGGGCTAGAGGACTAGCTAGCATCG |
| ATG6F-R  | ATGGGCTAGCGAGCATCAGTAGCAT |

### Western blotting

In the present study, the total protein was extracted from the specimens using the protein extraction kit (TAKARA) according to the instructions [12]. Then the antibody was diluted at 1:5000 according to the instructions provided by TAKARA, and western blotting was performed in accordance with the *Molecular Cloning Manual* [13].

### MDC staining

(1) First, the washing buffer in the kit was diluted using sterilized ultrapure water. (2) The cells were collected by centrifugation at 1000 g, and then the operation in (1) was repeated 3 times. (3) An appropriate amount of washing buffer was added and the number of cells was adjusted to  $10^6$ /mL. (4) 100  $\mu$ L of the above cells were added into a new EP tube, added with MDC dye, and mixed evenly. (5) The cells were incubated in a dark place for 30 min. (6) The cells were collected by centrifugation at 1000 g and washed with washing buffer for 3 times, and the supernatant was discarded. (7) The cells were resuspended in collection buffer, and 4  $\mu$ L cells were taken onto a glass slide. (8) Finally, the cells were observed under a fluorescence microscope (excitation wavelength: 355 nm, resistance filter wavelength: 512 nm), photographed and counted [14].

### Cell counting

An appropriate number of cells cultured were taken, ground, and resuspended in PBS (pH 7.4), followed by gradient dilution. Then they were smeared uniformly onto a plate and the colonies were counted [15].

### Data processing

All experimental results were statistically processed using the SPSS 20.0 software. Data were expressed as ( $\bar{x} \pm s$ ), and one-way analysis of variance was adopted for the multi-sample comparison of means, *t* test for the comparison

of means between two groups, and *q* test for the intergroup pairwise comparison.  $P < 0.05$  was considered significant.

## Results

### *mRNA expression levels of ATG13 and ATG6 in cells with overexpression and interference detected by fluorescence quantitative PCR*

To investigate the correlation between autophagy and drug resistance of mycobacteria, the cell lines with ATG13 and ATG6 interference and overexpression were constructed with Crisp/Case technique and verified by fluorescence quantitative PCR. As shown in **Figure 1**, the mRNA expression levels of ATG13 and ATG6 significantly declined in cell lines with interference compared with those in wild-type cell lines, showing significant differences compared with the control group ( $P < 0.05$ ). At the same time, the mRNA expression levels of ATG13 and ATG6 were significantly increased in cell lines with overexpression compared with those in wild-type cell lines, also showing significant differences compared with the control group ( $P < 0.05$ ). The above results indicated that the cell lines with ATG13 and ATG6 interference and overexpression were constructed successfully.

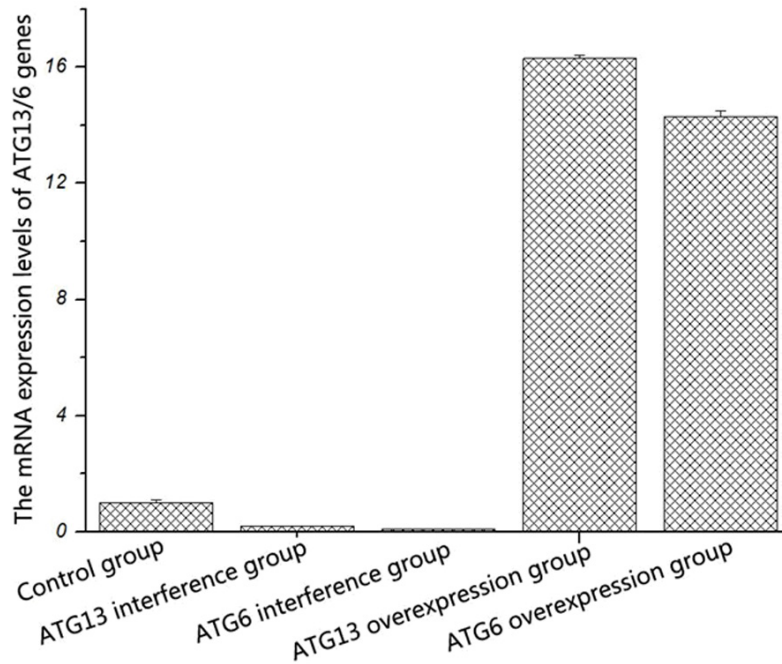
### *Protein expression levels of ATG13 and ATG6 in cells with overexpression and interference detected by western blotting*

The differences in the protein expression levels of ATG13 and ATG6 in cells with ATG13 and ATG6 overexpression and interference were detected. As shown in **Figure 2**, the protein expression levels of ATG13 and ATG6 in cells with ATG13 and ATG6 interference significantly declined compared with those in control cell lines, displaying significant differences compared with control group ( $P < 0.05$ ). At the same time, the protein expression levels of ATG13 and ATG6 in cells with ATG13 and ATG6 overexpression were significantly increased compared with those in control cell lines, also displaying significant differences compared with the control group ( $P < 0.05$ ).

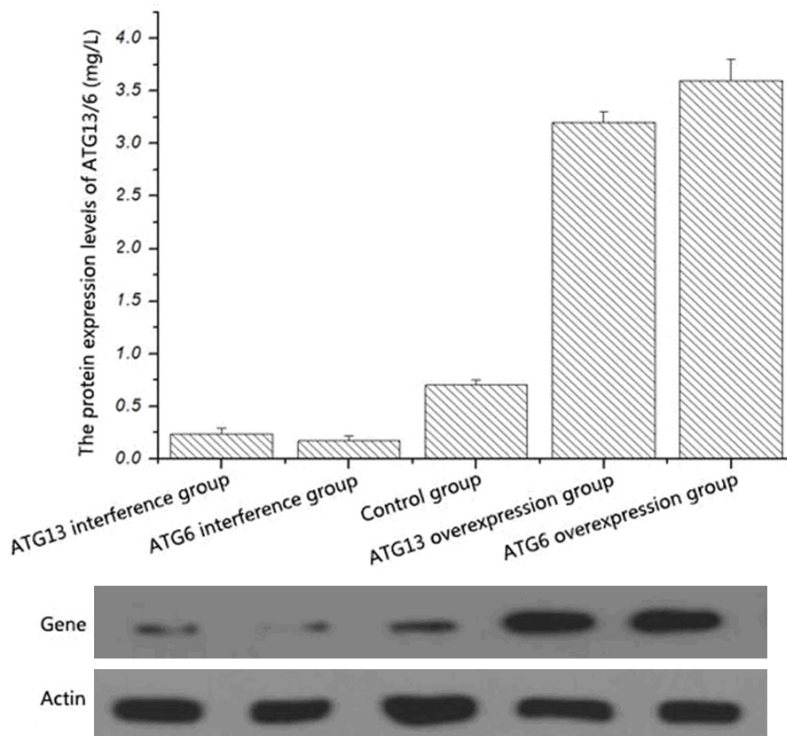
### *Autophagosomes in cells with ATG13 interference and overexpression observed by MDC staining*

To explore the correlation between ATG13 and autophagy, the autophagosomes in cells with

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**Figure 1.** mRNA expression levels of ATG13 and ATG6 in cells with overexpression and interference.



**Figure 2.** Protein expression levels of ATG13 and ATG6 in cells with ATG13 and ATG6 interference and overexpression.

ATG13 interference and overexpression were observed by MDC staining. As shown in **Figure**

**3**, the autophagy obviously declined in cell lines with ATG13 interference compared with that in control group, ( $P < 0.05$ ). At the same time, the autophagy was obviously higher in cell lines with ATG13 overexpression than that in control group ( $P < 0.05$ ). The above results suggest that the overexpression of ATG13 can significantly increase the autophagy.

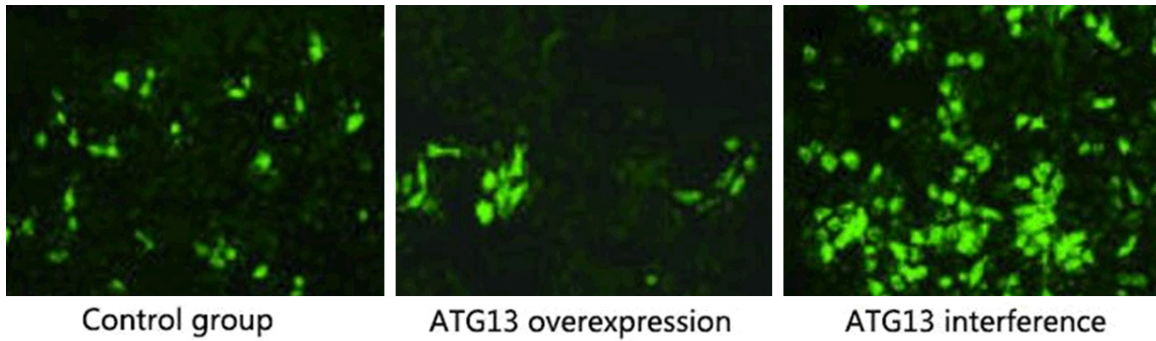
*Autophagosomes in cells with ATG6 interference and overexpression observed by MDC staining*

To explore the correlation between ATG6 and autophagy, the autophagosomes in cells with ATG6 interference and overexpression were observed by MDC staining. As shown in **Figure 4**, the autophagy obviously declined in cell lines with ATG6 interference compared with that in control group ( $P < 0.05$ ). At the same time, the autophagy was obviously higher in cell lines with ATG6 overexpression than that in control group ( $P < 0.05$ ). The above results, consistent with those in ATG13, suggest that the overexpression of ATG6 can significantly increase autophagy.

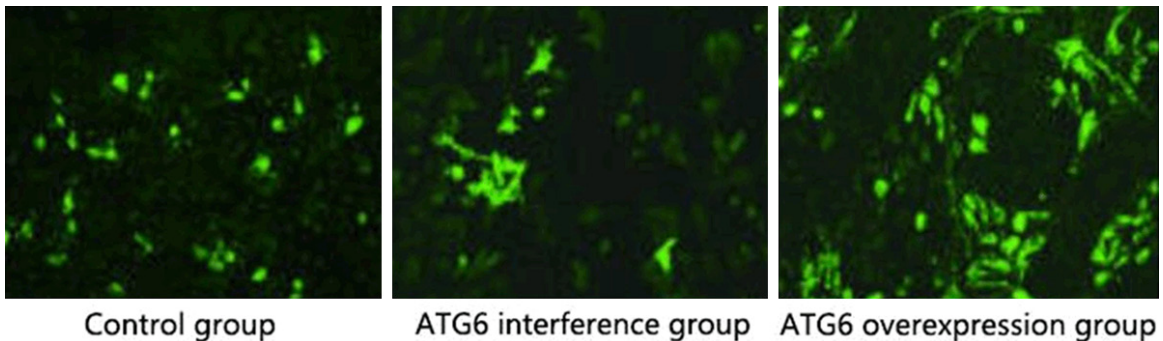
*Survival number of mycobacteria in cells with ATG13 interference and overexpression after different drug treatments*

To explore the correlation between ATG13 and drug resistance of mycobacteria, the survival number of mycobacteria in cells with ATG13 interference and overexpression after different drug treatment

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**Figure 3.** Autophagosomes in cells with ATG13 interference and overexpression, MDC staining.



**Figure 4.** Autophagosomes in cells with ATG6 interference and overexpression, MDC staining.

was detected. It can be seen from **Figure 5** that the number of mycobacteria in cell extract of cell lines with ATG13 interference after different drug treatments was increased compared with that in the control group ( $P < 0.05$ ). At the same time, the number of mycobacteria in the extract of cell lines with ATG13 overexpression after different drug treatments was decreased compared with that in the control group ( $P < 0.05$ ), indicating that the overexpression of ATG13 can significantly reduce the drug resistance of mycobacteria.

### *Survival number of mycobacteria in cells with ATG6 interference and overexpression after different drug treatment*

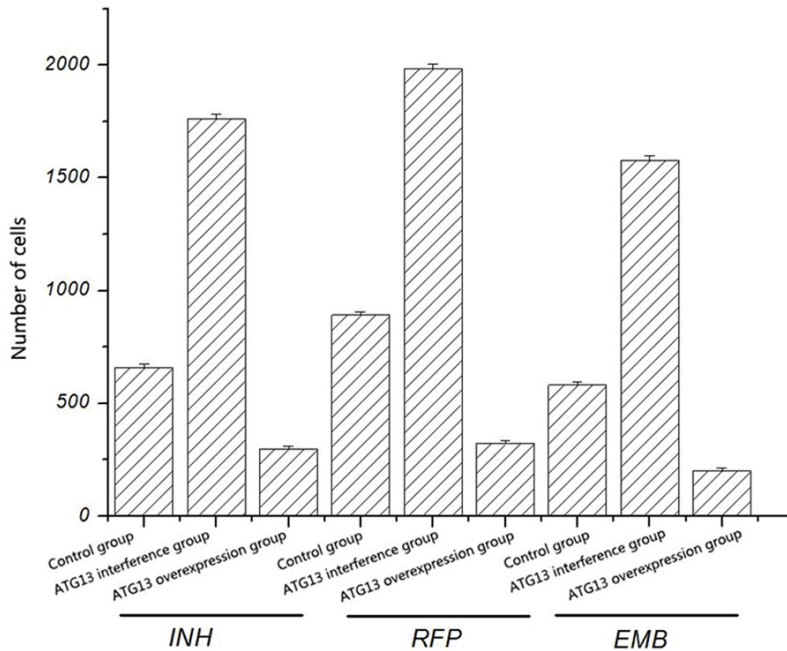
ATG6, an important gene that induces the fusion of autophagosomes with lysosomes, has an important correlation with autophagy. To explore the correlation between ATG6 and drug resistance of mycobacteria, the survival number of mycobacteria in cells with ATG6 interference and overexpression after different drug treatments was detected. It can be seen from **Figure 6** that the number of mycobacteria in

the extract of cell lines with ATG6 interference after different drug treatments was increased compared with that in control group ( $P < 0.05$ ). At the same time, the number of mycobacteria in the extract of cell lines with ATG6 overexpression after different drug treatments was decreased compared with that in control group, ( $P < 0.05$ ), indicating that overexpression of ATG6 can significantly reduce the drug resistance of mycobacteria.

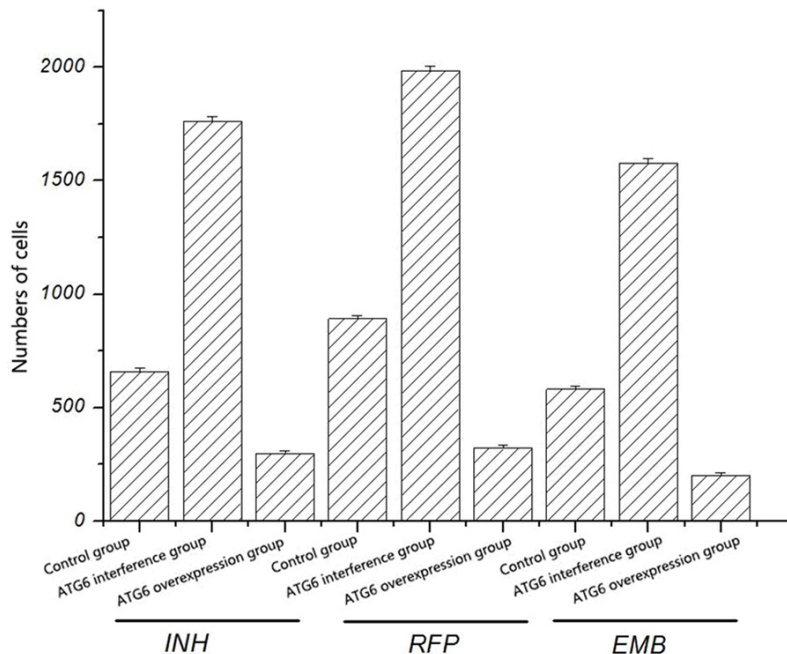
### **Discussion**

Acid-fast Mycobacterium tuberculosis, as the main pathogen of tuberculosis, plays an important role in the pathogenesis of tuberculosis. Studies have found that macrophages serve as host cells for Mycobacterium tuberculosis in animals, and the normal function of macrophages, important immune cells that remove exogenous foreign bodies in the body, is significant in animals. The mycobacteria invading macrophages can inhibit the ATP-dependent proton pump in the phagosome membrane surface vesicles in macrophages to inhibit the acidification of phagosomes, thereby suppress-

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**Figure 5.** Surviving number of mycobacteria in cells with ATG13 interference and overexpression after different drug treatments.



**Figure 6.** Surviving number of mycobacteria in cells with ATG6 interference and overexpression after different drug treatments.

ing the fusion of phagosomes with lysosomes, ultimately producing drug resistance of mycobacteria. Therefore, exploring how to improve the body's resistance to *Mycobacterium tuberculosis* and remove the *Mycobacterium tuber-*

*culosis* in macrophages has important significance in keeping the normal function of the body [16]. In recent years, with the significant increase in the drug resistance of mycobacteria, the effects of many antibiotics on mycobacteria have declined in different degrees [17, 18]. Therefore, it has important theoretical and practical significance to explore new mechanisms for the resistance to mycobacteria. As an important means for cells to maintain the normal physiologic metabolism, autophagy can not only remove the damaged organelles and mis-coded proteins in cells [19], but also plays an important role in scavenging the pathogens in the body and keeping the normal physiologic functions of cells [20]. In the present study, the cell lines with ATG13 and ATG6 interference and overexpression were constructed with Crisp/Cas technique and verified at mRNA and protein levels. The results showed that after ATG13/6 interference, the autophagy was significantly reduced, and the fusion of phagosomes with lysosomes was weakened. After the above cells with ATG13/6 interference were infected with mycobacteria and treated with INH (0.05 mg/L), RFP (0.4 mg/L) and EMB (25 mg/L), they were counted. It was found that the ATG13/6 interference could significantly improve the drug resistance of mycobacteria, indicating that the decline in the expression levels of autophagy-related genes ATG13/6 in macrophages can attenuate the autophagy of macrophages to reduce its ability to scavenge myco-

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bacteria, ultimately increasing the drug resistance of mycobacteria. Also, after the cells with ATG13/6 overexpression were infected with mycobacteria and treated with INH (0.05 mg/L), RFP (0.4 mg/L) and EMB (25 mg/L), they were counted. It was found that the ATG13/6 overexpression could obviously reduce the drug resistance of mycobacteria, suggesting that the up-regulation of the expression levels of autophagy-related genes ATG13/6 in macrophages can promote autophagy of macrophages to enhance its ability to scavenge mycobacteria, ultimately reducing the drug resistance of mycobacteria.

### Conclusion

In conclusion, the autophagy-related genes, such as ATG13/6, can degrade the mycobacteria invading cells through increasing the formation of autophagosomes and promoting fusion of autophagosomes with lysosomes, thereby reducing the drug resistance of mycobacteria. However, the signaling pathway through which the autophagy-related genes act on mycobacteria remains unclear, and the mechanism of action of different ATGs in mycobacteria, namely how they identify the mycobacteria, is also unclear. These are important directions in future research.

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### Disclosure of conflict of interest

None.

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