UVRAG-Knockdown Regulates Mitochondrial Autophagy in Chronic Myeloid Leukaemia Cells by Targeting BNIP3L

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Research Article

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Abstract

Objective: To explore whether UVRAG regulates mitochondrial autophagy via BNIP3L in K562 cells

Material and methods: We designed various assays to verify the relation between UVRAG and BNIP3L, we estabilished a mitochondrial autophagy model of K562 cells by CCCP, a mitochondrial autophagy inducer, and regulated the expression of UVRAG by cells transfection. Then we detected the expression of the BINP3L and autophagy-related proteins LC3-II/LC3- and P62 by Western blot. The changes of ROS, mitochondrial mass, and mitochondrial membrane potential (MMP) were detected by flow cytometry technology.

Results: We found that CCCP could induce K562 cells mitochondrial autophagy, along with the change of MMP, mitochondrial mass and accumulation of ROS, also our experiment proved that UVRAG-Knockdown could reverse this phenomenon. Investigating the pathway of mitochondrial autophagy revealed UVRAG knockdown was accompanied by a decrease in BNIP3L and LC3 expression, a increase in P62 during mitochondrial autophagy.

Conclusion: In our study, the results suggested that UVRAG may regulate mitochondrial autophagy of K562 cells via targeting BINP3L, which may be a potential target for the treatment of CML.

Introduction

Chronic myeloid leukaemia (CML) is clonal myeloproliferative malignancy derived from bone marrow haematopoietic stem cells. It has a high incidence rate and poor prognosis. Some patients have a relapse, accelerated and acute transformation phase (1, 2). Therefore, searching for new treatment methods has profound significance for the clinical treatment and prognosis for CML.

Autophagy is a highly conserved biological process in eukaryotes. It maintains cell stability by degrading and recycling toxic substances such as damaged organelles and metabolites (3). Mitochondrial autophagy is selective autophagy that can eliminate injured mitochondria and plays an important role in maintaining mitochondrial function and cell homeostasis (4). UVRAG is a tumour-suppressor gene involved in autophagy. It can suppress the development of cancer by regulating autophagy (5, 6). Our previous study showed that UVRAG might promote the autophagy of leukaemia cells (7). However, whether UVRAG is involved in mitochondrial autophagy in CML cells and its mechanism has not been investigated. In this study, we established a mitochondrial autophagy model in K562 cells and explored the mechanism of mitochondrial autophagy regulated by UVRAG to provide experimental clues for finding a new therapy.

Materials And Methods

Cell line and culture.

The CML cell line (K562) was purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. The cells were grown in PRMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and contained 10% foetal bovine serum and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in a saturated humidified incubator with 5% CO2 at 37℃.

A transient transfection cell line with UVRAG-Knockdown and a mitochondrial autophagy model was established.

Transfection of cells with siRNA was used to knock down the UVRAG gene, and a scrambled shRNA was used as a negative control. CCCP, a mitophagy inducer, was used to induce mitophagy. Cells in the logarithmic growth phase were seeded in a 6-well plate. The transfection method was carried out in strict accordance with Lipofectamine 3000 specifications. Then, cells were incubated at 37℃ with 5% CO2. After 24 or 48 hours, cells were collected for qPCR or Western blot.

Detecting cell proliferation by CCK-8 assay.

K562 cells in the logarithmic growth phase were seeded in a 96-well plate (1 × 104 cells/well), and different concentrations of CCCP (0, 10, 20, 50, 100 μmol/L) were added. The proliferation rate was detected by CCK-8 assay according to the manufacturer's instructions (Vazyme Biotech Co., Ltd). Ten ul CCK-8 reagent was added to each well after 0, 6, 12, 24, and 48 hours of cell culture. Absorbance at 450 nm was measured after incubation for two hours at 37℃.

Experiment group.

K562 cells in the logarithmic growth phase were collected and seeded in a 6-well plate (1 × 104 cells/well), which were divided into the NC group, the UVRAG siRNA group, the UVRAG siRNA + CCCP group, and the CCCP group. Cells in the UVRAG siRNA group and the UVRAG siRNA + CCCP group were transfected with siRNA. After 36 hours, cells in the UVRAG siRNA + CCCP group and the CCCP group were treated with CCCP, and the final concentration of CCCP was 50 μmol/L. After continuous culture for 12 hours, cells were collected for detection.

Detection of mitochondrial mass and mitochondrial membrane potential (MMP) by flow cytometry.

Cells in each group were resuspended in 0.5 ml medium, and 0.5 ml JC solution was added. All were mixed fully and incubated in a 37℃ incubator for 20 minutes. During cell incubation, JC-1 dyeing buffer (1×) was prepared, then cells were centrifuged at 600 g and 4℃ for four minutes. The supernatant was discarded. Cells were washed with the dyeing buffer twice. Next, 300 μl JC-1 dyeing buffer (1×) was added to each group. MMP was detected by flow cytometry. Cells were resuspended in a 12-well plate when mitochondrial mass was detected. Five μmmol/L NAO fluorescence probe was added to cells in each group and incubated at 37℃ for 30 minutes and washed with PBS twice to remove the remaining fluorescent probes that did not enter the cells. Mitochondrial mass was detected by flow cytometry.

Detection of ROS by flow cytometry.

According to the manufacturer's instructions, ROS was detected by the Reactive Oxygen Species Assay Kit (Shanghai ZCIBIO Technology Co., Ltd). All cells were harvested and added with 500 μl DCFH-DA diluted to 10 μmol/L in a serum-free medium. They were then incubated for two hours at 37℃ and washed with PBS three times. All samples were placed under a flow detection.

Verification of the transfection effect of UVRAG gene by qPCR.

Cells were washed with PBS, and TRIzol reagent (Vazyme Biotech Co., Ltd.) was used to extract total RNA according to the manufacturer's instructions. A spectrophotometer was used to detect RNA concentrations of 1500 ng/μl and A260 nm/A280 nm between 1.8–2.0. Subsequently, the PrimeScript RTPCR Kit (Vazyme Biotech Co., Ltd.) was used to synthesise cDNA, and the reaction conditions were as follows: 37℃, 15 minutes, and 80℃, five seconds. RT-qPCR was performed using a One-Step SYBR PrimeScript RT-qPCR Kit (Yeasen Biotechnology (Shanghai) Co., Ltd.) with the following thermocycling conditions: 95℃ for five minutes, 95℃ for ten seconds, 55–60℃ for 20 seconds, 72℃ for 20 seconds, a total of 40 cycles. Relative expression was calculated by the 2 - △△CT method.

Western blot.

Cells were lysed with a lysis buffer (KeyGEN BioTECH) for 30 minutes on ice. The supernatant was collected by centrifugation at 14,000 rpm for 30 minutes at 4℃. The BCA protein assay kit was used to detect protein concentrations. SDS-PAGE: protein (50 μg/lane) were separated using a 10% SDS polyacrylamide gel, then transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk for two hours at room temperature and incubated overnight at 4℃ with the anti-UVRAG antibody (dilution, 1:1,000; rabbit YN1925; Immunoway), the antiLC3 antibody (dilution, 1:1,000; rabbit 12741; CST), the antiP62 antibody (dilution, 1:1,000; rabbit 8025; CST), the antiBNIP3L antibody (dilution, 1:1,000; rabbit YN2077; Immunoway) and the antiβ-actin antibody (dilution, 1:1,000; mouse 60008; Immunoway). They were then incubated with HPR Goat Anti-Rabbit IgG (dilution, 1:5,000; 40295; Immunoway) for one hour at room temperature. Densitometry was performed using Image J.

Statistical analysis.

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, Inc.). The data were presented as the mean ± SEM. The statistical comparison between the two groups was performed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CCCP inhibits the proliferation of K562 cells.

K562 cells were treated with different concentrations of CCCP for 6, 12, 24 and 48 hours, and the proliferation rates of K562 cells were measured using CCK-8 assay. The results showed that the proliferation rates of K562 cells treated with 50 μmol/L CCCP for 12 hours was about 55%, and 50 μmol/L CCCP for 12 hours was the most suitable (Fig. 1).

Transfection of cells with siRNA knockdown significantly affects the expression of the UVRAG gene.

To explore whether the transfection of cells with siRNA silenced UVRAG, we detected the expression of UVRAG protein and mRNA by Western blot (Fig. 2A)and qPCR (Fig. 2B). The results suggested that the expression of UVRAG protein and mRNA declined sharply, which was consistent.

The UVRAG-Knockdown of K562 reverses the decrease of MMP.

Flow cytometry analysis results revealed no significant difference between the MMP of the NC group (96.7 ± 1.4) and the MMP of the UVRAG siRNA group (97.8 ± 0.1). Compared with the UVRAG siRNA + CCCP group (88.6 ± 5.4), the MMP of the CCCP (76.8 ± 3.) group declined significantly ($P < 0.05$). The difference was statistically significant (Fig. 3A, B).

The UVRAG-Knockdown of K562 cells inhibits the reduction in mitochondrial mass.

Cells in each group were treated with 5 μmol/L NAO for 30 minutes. Flow cytometry analysis results showed that there was no significant difference in the mitochondrial mass between the NC group and the UVRAG siRNA group ($P > 0.05$). This result was considered to show that the mitochondrial mass was relatively complete. Compared with the UVRAG siRNA + CCCP group, the decrease of mitochondrial mass in the CCCP group was statistically significant ($P < 0.05$) (Fig. 4A, B).

UVRAG-Knockdown could prevent the increase of ROS.

Cells in each group treated with 10 μmol/L DCFH-DA probes were incubated for 20 minutes and washed with PBS three times. Cells were detected by flow cytometry (Fig. 5A). The results showed that the level of ROS between the UVRAG siRNA group and the NC group had no significant difference ($P < 0.05$). Compared with the UVRAG siRNA + CCCP group, the ROS in the CCCP group greatly increased, and the difference was statistically significant ($P < 0.05$) (Fig. 5B). Therefore, UVRAG-Knockdown could inhibit the production of ROS.

UVRAG-Knockdown reduces the level of autophagy via BNIPL3.

UVRAG-Knockdown regulated mitochondrial autophagy via BNIP3L

The expression of LC3, P62, and BNIP3L in each group was detected by western bloting (Fig. 6A and 7A). The Western blot results demonstrate that compared with the CCCP group, the expression of LC3 and BNIP3L decreased significantly in the UVRAG siRNA + CCCP group (Fig. 6B, 7B), and the expression of P62 increased significantly ($P < 0.05$) (Fig. 7B). There were no obvious differences between the UVRAG siRNA group and the CCCP group ($P > 0.05$). Collectively, these results suggested that UVRAG-Knockdown regulated mitochondrial autophagy via BNIP3L.

Discussion

Chronic myeloid leukaemia is one of the main types of leukaemia. It is hard for traditional chemotherapy drugs to improve the remission rate of patients with leukaemia. There is an urgent need to seek an effective treatment method. Thus, it is particularly important to explore the new molecular mechanism of the occurrence and development of CML.

Autophagy mediates the degradation and circulation of lysosome-dependent cytoplasmic substances, which constitutes the quality control programme of cells and makes cells react correctly to stress. Autophagy usually plays a role in tumour suppressors (8). The absence of autophagy genes often leads to the downregulation of autophagy activity, accompanied by DNA damage that could not be repaired effectively, resulting in the development of tumours (9). UVRAG is a tumour-suppressor gene that could activate autophagy and plays an important role in DNA damage response. UVRAG forms different complexes with BECN1 and C-Vps at different stages of autophagy, which enhances the total flux of autophagy degradation. The function of UVRAG is mainly to act as a pivotal regulator of autophagy and chromosomal stability, and its mutation and deletion are involved in the occurrence and development of tumours (10). Moreover, UVRAG also promotes DNA double-strand breakthrough binding and activating DNA-pk in non-homologous end connection, and the destruction of UVRAG could increase genetic instability and cell sensitivity to radiation (10). Wu et al. found that UVRAG, BRCA1, BECN1, CCND and PTEN participated in the occurrence of breast during different periods, and their abnormal expression might play an important role in the occurrence of human breast cancer through the disorder of mRNA expression (11). Thus, we can see that UVRAG, as a tumour-suppressor gene, is closely related to tumorigenesis and progression, which can inhibit the formation and proliferation of tumour cells by upregulating autophagy. Mitochondrial autophagy, a selective autophagy, plays an important role in maintaining mitochondrial quality control and homeostasis (12). Mitochondria is an important site of ROS production. ROS is at a low level when the function of mitochondria is normal; conversely, when mitochondria are dysfunctional, the increase of ROS can lead to DNA damage and accelerate the metastasis and development of cancer by promoting abnormal gene expression (13). Therefore, we can provide experimental clues for researching new treatment methods for CML by studying the mechanism of UVRAG participating in mitochondrial autophagy of K562 cells.

In the present study, we established a mitochondrial autophagy model with 50 μmol/L CCCP-treated K562 cells to further explore whether UVRAG was involved in mitochondrial autophagy and its mechanism in K652 cells. First, UVRAG siRNA was transfected into K562 cells. Subsequently, our results demonstrated that UVRAG could be knocked down effectively after transfection.

Mitochondrial autophagy is a process that selectively removes damaged mitochondria and maintains intracellular homeostasis through autophagy mechanisms. Mitochondrial autophagy would activate inflammasomes such as NLRP3 to clear damaged mitochondria and inhibit cells from dying when the invasion of viruses or bacteria leads to mitochondrial DNA damage and a large number of ROS. The deficiency of mitochondrial autophagy is closely related to hereditary diseases, cancer, diabetes, and others (14). Wei et al. found that matrine regulated mitochondrial autophagy and promoted liver cancer cells to die through the PINK1/Parkin pathway (15). Because the occurrence of mitochondrial autophagy

is accompanied by the decrease of MMP and mitochondrial quality (16), we sensinged the level of MMP and mitochondrial mass by JC-1 fluorescent probe and fluorescence indicator NAO respectively. Flow cytometry analysis results demonstrated that UVRAG-Knockdown could significantly reverse the decrease of MMP and mitochondrial mass in K562 cells. When there is nutritional starvation, traumatic injury and other pathological conditions, the balance between the oxidation and antioxidation systems is disordered, and accumulation of ROS induces mitochondrial autophagy (17). In this study, the ROS content in K562 cells was analysed by flow cytometry. The results showed that UVRAG-Knockdown could significantly reduce the accumulation of ROS, which suggested that UVRAG might be involved in mitochondrial autophagy of K562 cells.

At present, the research on the pathways of mitochondrial autophagy includes autophagy mediated by mitochondrial autophagy receptors (such as BNIP3L, BINP3, FUNDC1) and the PINK1/Parkin pathway (18). LC3, a standard marker for autophagosomes as a mammalian Atg8 homologue, is a soluble protein. In the formation of autophagosomal membranes, LC3-I, which is cytosolic and a soluble form of LC3, is conjugated with phosphatidylethanolamine (PE) through two consecutive ubiquitylation-like reactions catalyzed by the E1-like enzyme Atg7 and the E2-like enzyme Atg3 to LC3-II (membrane-bound form of LC3). During the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is also degraded by lysosomal proteases. Therefore, the changes of cellular LC3-II level are connected to the dynamic turnover of LC3-II via the lysosome, i.e., autophagic activity (19). BNIP3L, located in the outer mitochondrial membrane (OMM), is a selective mitochondrial autophagy receptor, which can cause cell death mediated by mitochondria. At its N-terminal, an LIR motif is exposed in the cytoplasm, which can bring mitochondria into autophagosome by 0binding to the Atg8 homologous LC3/GABARAP protein (20). LIR phosphorylation is essential for BNIP3L. It helps enhance the interaction with LC3 and BNIP3L to start and develop BNIP3L-dependent mitochondrial autophagy properly (21, 22). These studies indicate that BNIP3L is an important component of mitochondrial autophagy and that a BNIP3L-absence would generate a decrease in mitochondrial autophagy. P62, an autophagy substrate, also participates in selective autophagy and promotes autophagy by binding to LC3 through the LIR domain (23). Next, the expressions of BNIP3L, LC3, and P62 were detected by Western blot. The analysis results show that UVRAG-Knockdown could markedly downregulate the expressions of BNIP3L, LC3 and P62. Overall, UVRAG might modulate mitochondrial autophagy of K562 cells through the BNIP3L-mediated mitochondrial autophagy receptor pathway.

In conclusion, the present study showed that UVRAG might regulate mitochondrial autophagy of CML through BNIP3L-mediated mitochondrial autophagy receptor pathways. This research provided novel insights for the treatment of CML. However, mitochondrial autophagy is a complex process, which may be mediated by multiple pathways, and further research is needed.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

CYL, YMM performed the experiments and data analysis. XCY conceived and designed the study. CYL and YMM confirmed the authenticity of all the raw data. XQZ and YL reviewed and edited the manuscript. CYL and YMM participated in data analysis and draft writing. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Ethics approval and consent to participate

Not Applicable. This study did not involve human participants and animals

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

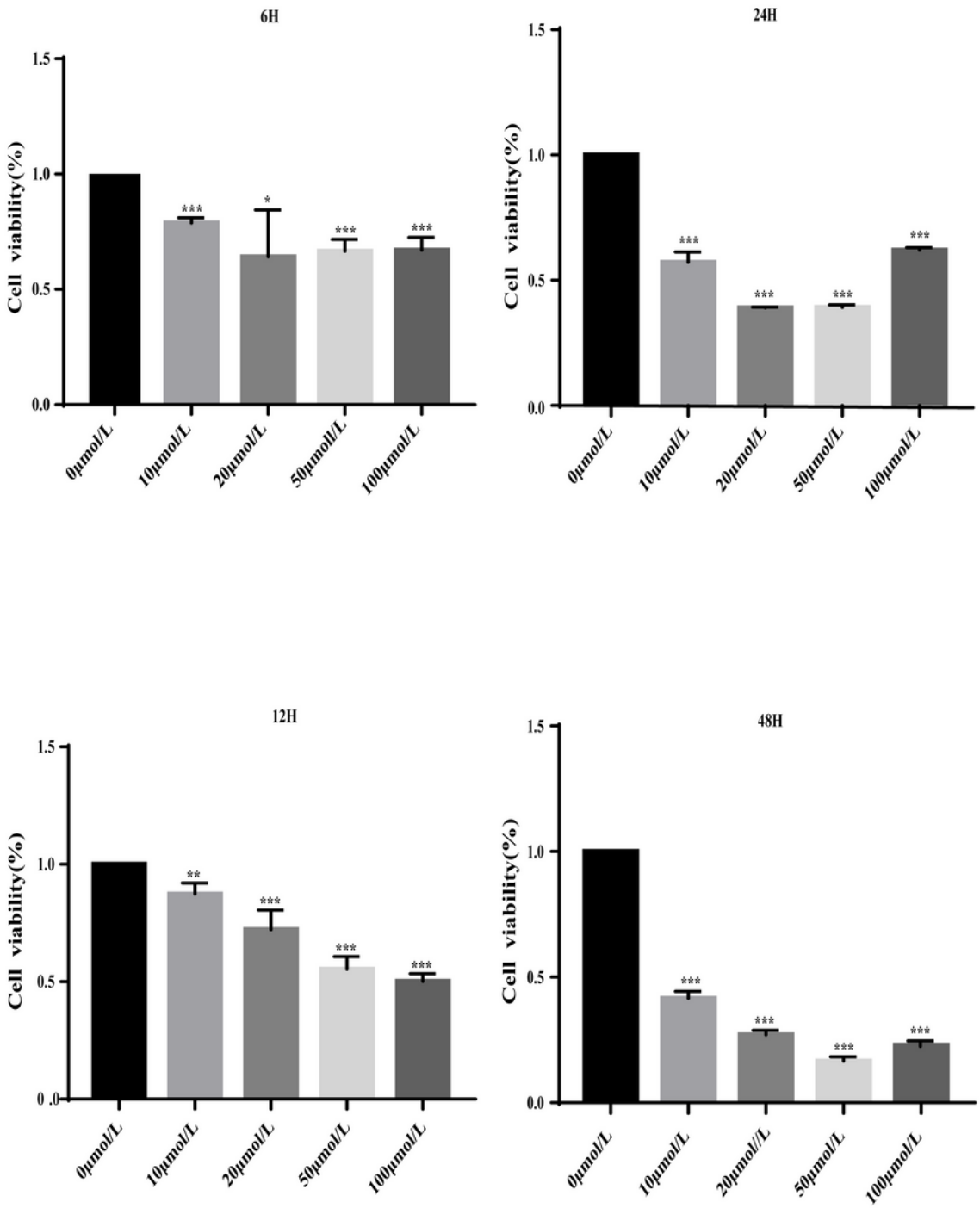

Figure 1

CCCP suppresses the proliferation of K562 cells. K562 cells treated with different concentrations of CCCP for 6h, 12h, 24h, 48h, CCK-8 assay indicated that 50 μmol/L CCCP for 12 hours was the most suitable, *P < 0.05, **P < 0.01, ***P < 0.001.

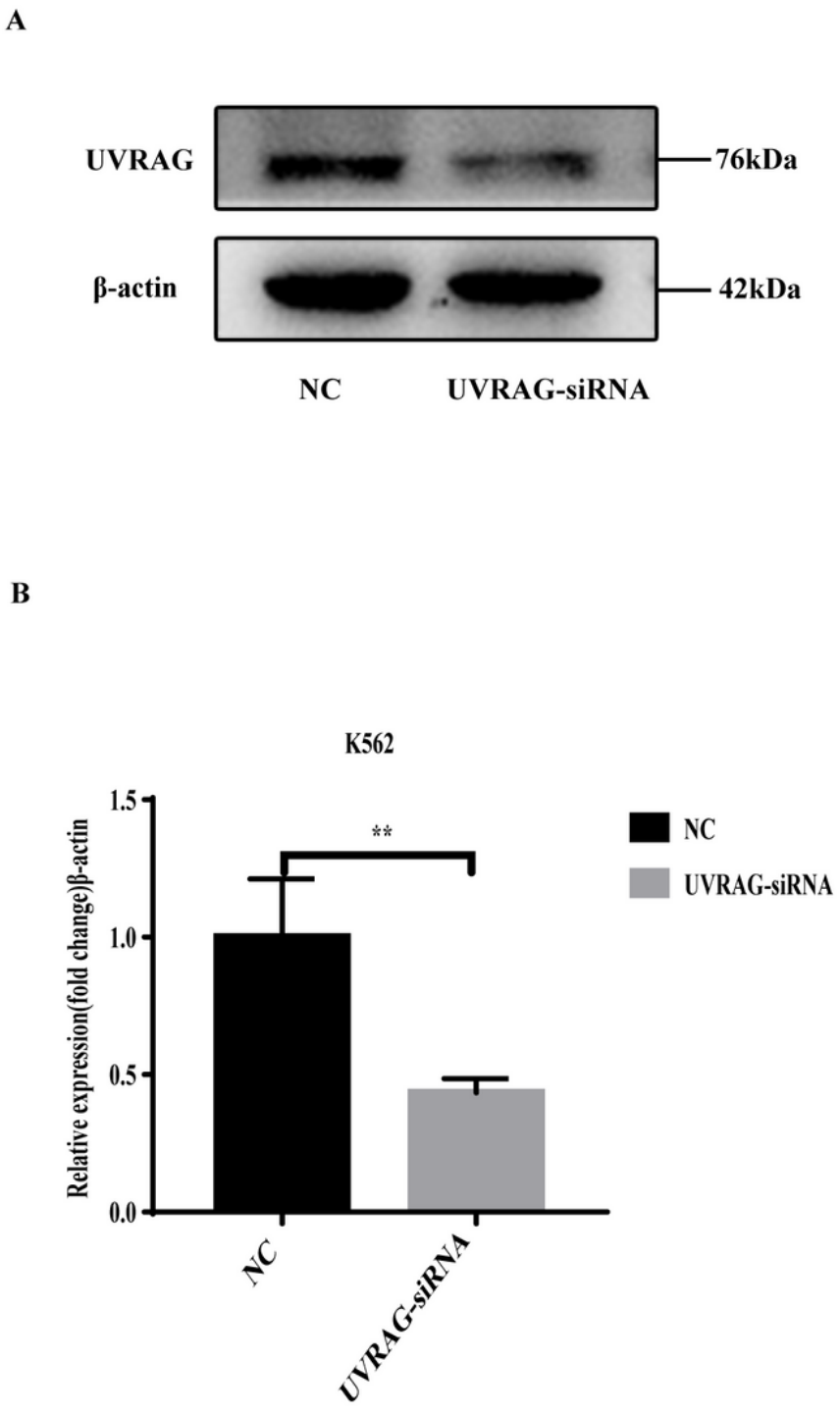

Figure 2

Transfection of cells with siRNA could knockdown expression of UVRAG. A: The expression of UVRAG after tranfection of siRNA was decreased compared with control cells. B: The relative expression of

UVRAG mRNA after tranfection of siRNA was decreased compared with control cells.*P < 0.05, **P < 0.01, ***P < 0.001.

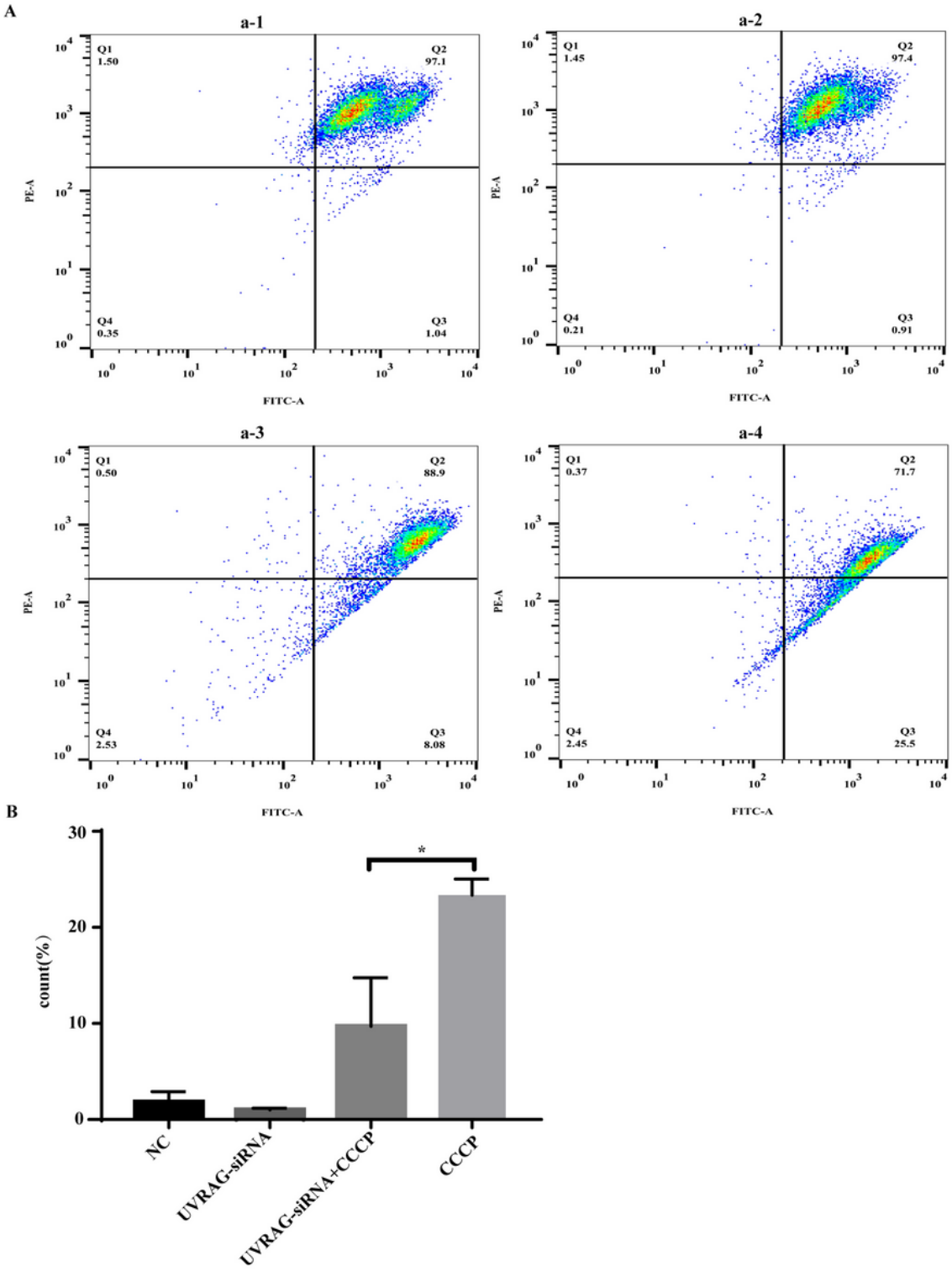

Figure 3

The UVRAG-knockdown of K562 could reversed the decreasing of mitochondrial membrane potential. A: The changes of MMP in each group was detected by flow cytometry. a-1: NC group; a-2: UVRAG siRNA group; a-3: UVRAG siRNA+CCCP group; a-4: CCCP group. B: Significantly decreased the mitochondrial

membrane potential in UVRAG siRNA+CCCP group Compared to CCCP group(*P 0.05). MMP: mitochondrial membrane potential.

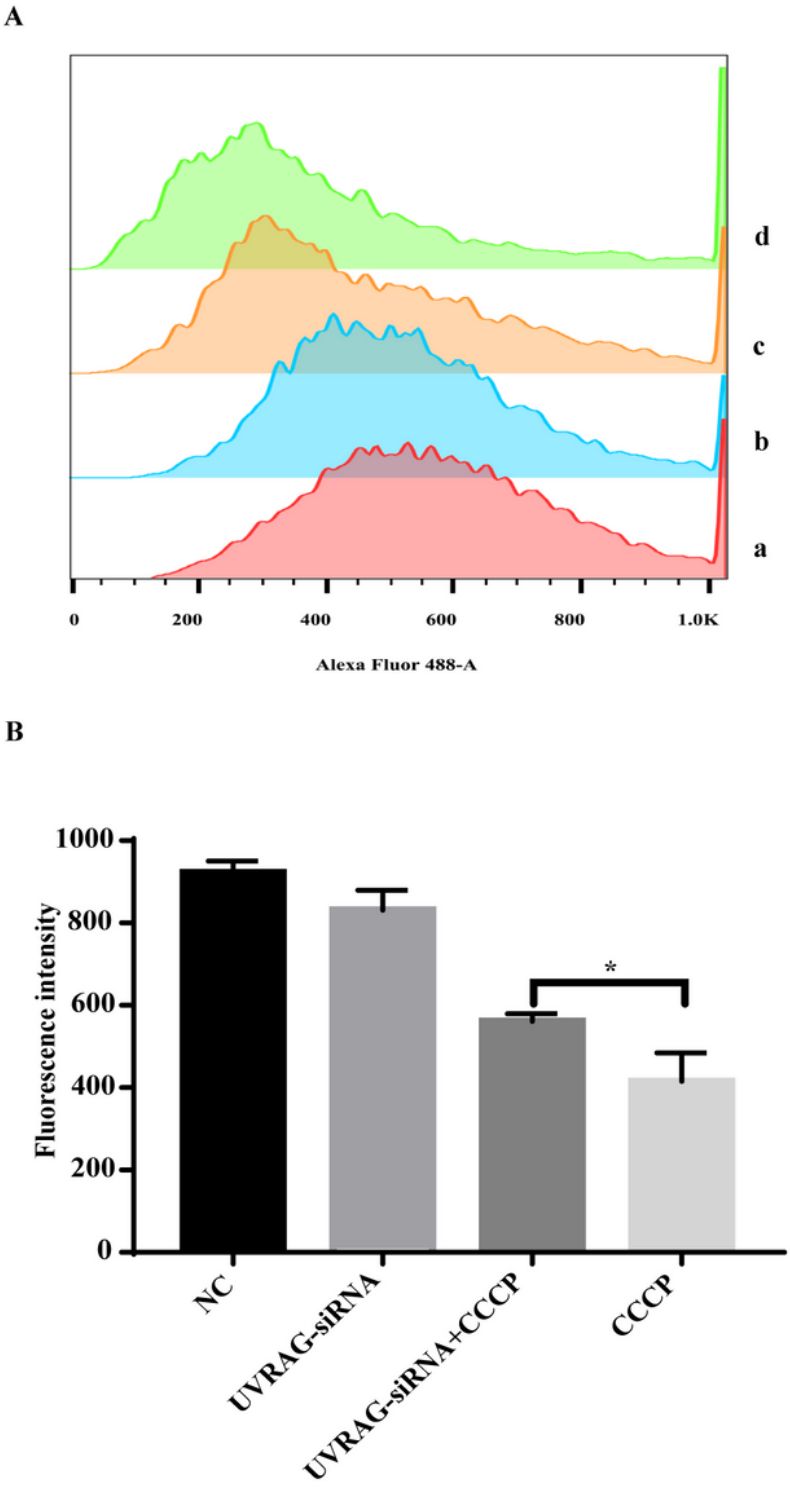

Figure 4

The UVRAG-Knockdown of K562 cells inhibited the reduction of mitochondrial mass. A: Cells in each group were collected and treated with JC-1 dyeing buffer (1×), and mitochondrial mass was detected by flow cytometry. a: NC group; b: UVRAG siRNA group; c: UVRAG siRNA+CCCP group; d:CCCP group. B: The

UVRAG-Knockdown of K562 cells after CCCP treatment reversed the decrease of MMP(*P 0.05). MMP: mitochondrial membrane potential.

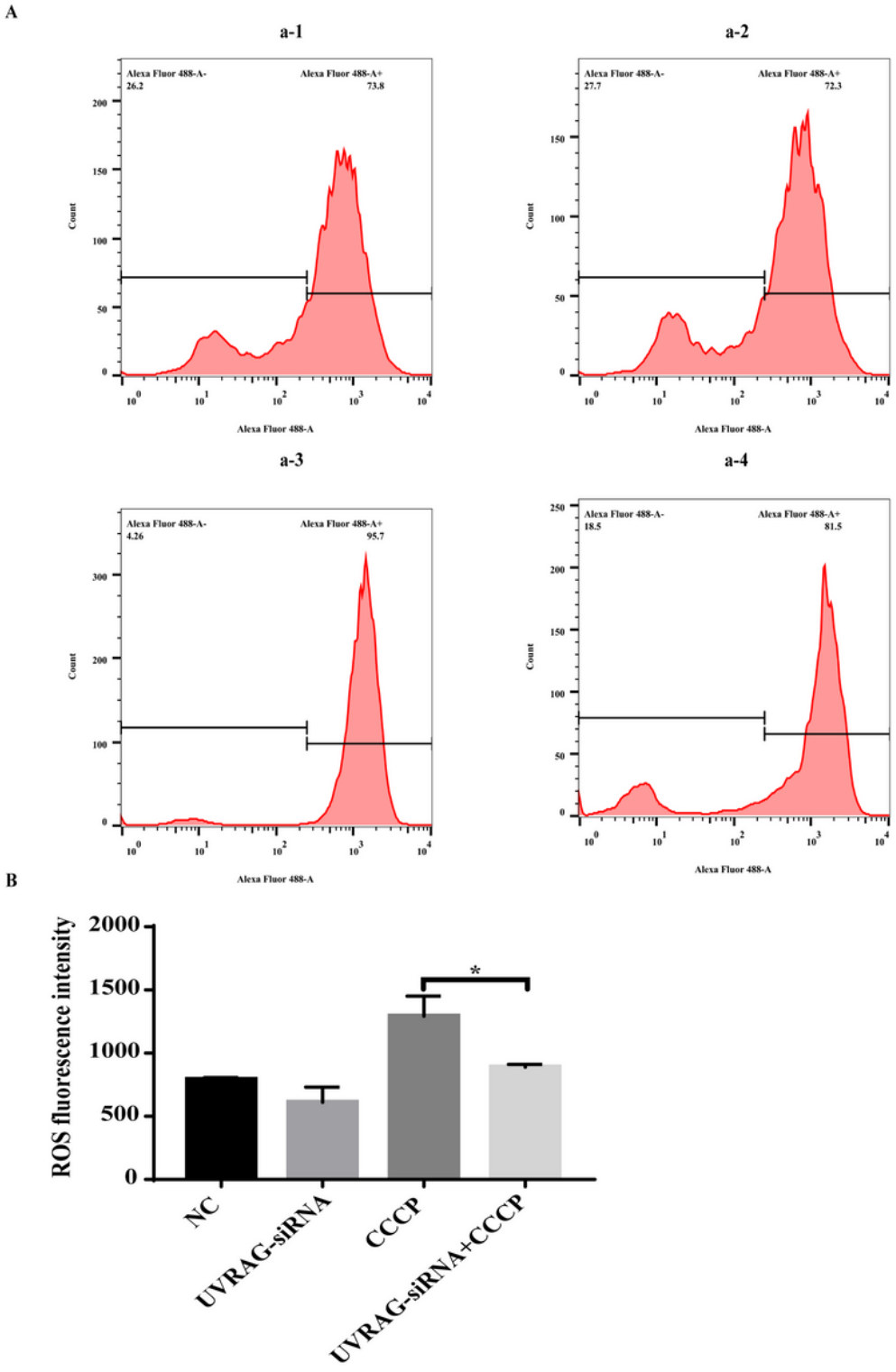

Figure 5

UVRAG-Knockdown inhibited the production of ROS. A: ROS in each group was detected by flow cytometry. a-1: NC group; a-2: UVRAG siRNA group; a-3: UVRAG siRNA + CCCP group; a-4: CCCP group. B:

The UVRAG-Knockdown of K562 cells after CCCP treatment significantly reduced the increasing of ROS(*P 0.05).

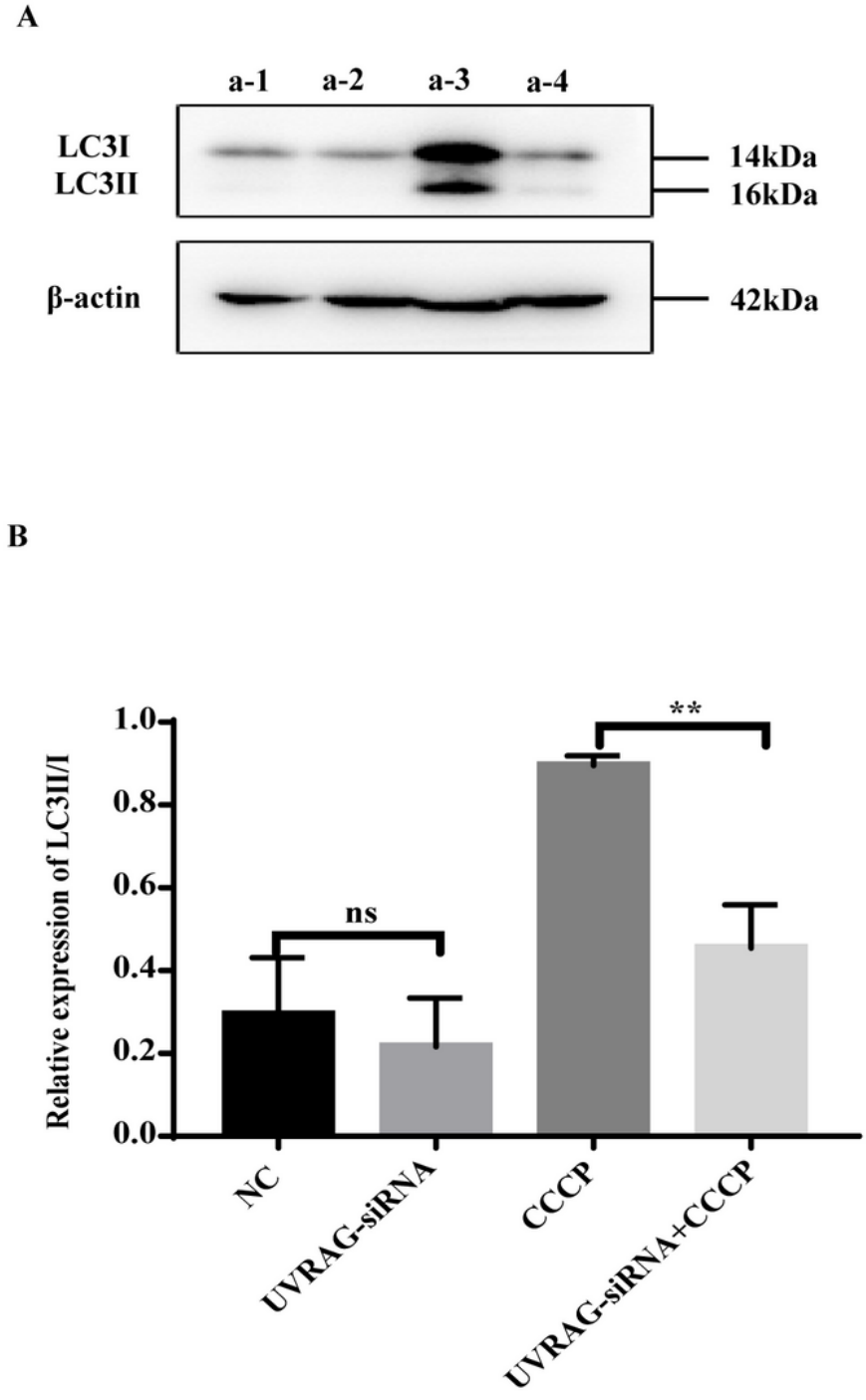

Figure 6

UVRAG-Knockdown regulated mitochondrial autophagy through BNILP3. A: Detections of LC3I and LC3II expression were performed by western blot analysis. a-1: NC group; a-2: UVRAG siRNA group; a-3: UVRAG

siRNA + CCCP group; a-4: CCCP group. B: The relative expression of LC3II/I in UVRAG siRNA + CCCP group was decreased compared to CCCP group(**P < 0.01).

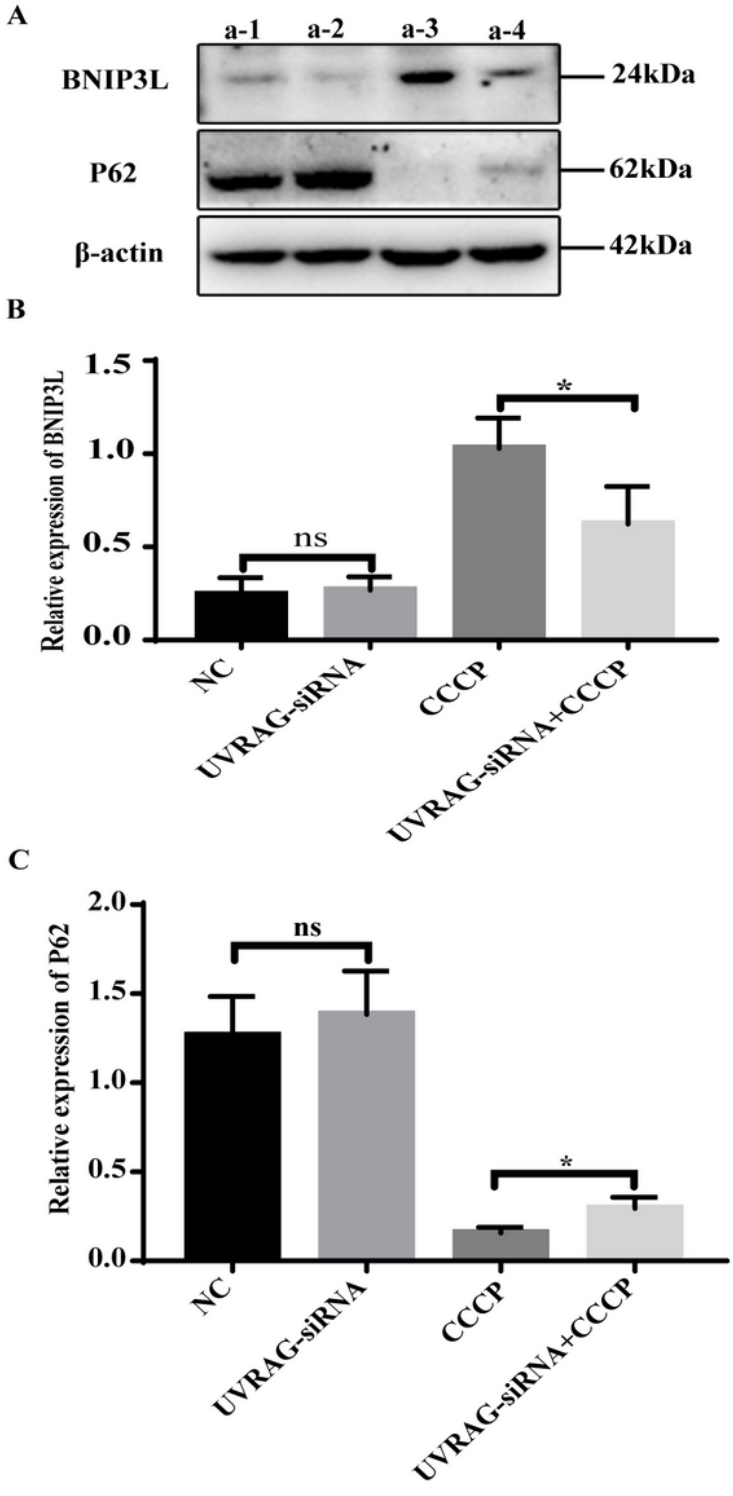

Figure 7

UVRAG-Knockdown regulated mitochondrial autophagy through BNILP3. A: Detections of BNIP3L and P62 expression were performed by western blot analysis. a-1: NC group; a-2: UVRAG siRNA group; a-3: CCCP group; a-4: UVRAG siRNA + CCCP group. B: The relative expression of P62 in UVRAG siRNA + CCCP

group was decreased compared to CCCP group and the relative expression of P62 in UVRAG siRNA + CCCP group was increased compared to CCCP group(*P < 0.05).