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Original Paper

Knockdown of Linc00515 Inhibits Multiple Myeloma Autophagy and Chemoresistance by Upregulating miR-140-5p and Downregulating ATG14

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Key Words
Linc00515 • ATG14 • miR-140-5p • Multiple myeloma • Autophagy • Chemoresistance

Abstract
Background/Aims: The purpose of our experiments was to investigate the targeting relationship of linc00515, miR-140-5p and *ATG14* and to explore the roles of linc00515, miR-140-5p and *ATG14* in autophagy and chemoresistance of melphalan-resistant multiple myeloma cells. ***Methods:*** Plasmids that could interfere with the expression of linc00515 and *ATG14* were loaded into myeloma cells, which were cultured with melphalan. MTT assay and flow cytometry analysis were utilized to investigate the effect of linc00515, miR-140-5p and *ATG14* on the resistance of myeloma cells. QRT-PCR was used to determine the levels of mRNAs. Western blot was utilized to explore the level of ATG14 and autophagy-related proteins. Dual luciferase assay was utilized to explore the targeting relationship between linc00515, miR-140-5p and *ATG14*. GFP LC3 fluorescence assay was conducted to study the autophagy of cells. ***Results:*** The expression of linc00515 and *ATG14* were significantly higher in melphalan-resistant myeloma cells. Knockdown of linc00515 and *ATG14* led to decreased autophagy and chemoresistance of melphalan-resistant myeloma cells. The forced expression of miR-140-5p suppressed autophagy and chemoresistance of melphalan-resistant myeloma cells. ***Conclusion:*** Linc00515 enhanced autophagy and chemoresistance of melphalan-resistant myeloma by directly inhibiting miR-140-5p, which elevated *ATG14* level.

D. Lu and C. Yang contributed equally to this work.

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Introduction

Multiple myeloma (MM) is a kind of neoplastic plasma-cell disorder, characterized by the immersion of malignant plasma cells into bone marrow and still incurable [1, 2]. The survival terms of patients with MM range from a few weeks to more than 10 years with the five-year survival rate of 40 %. Etiologies and potential molecular mechanisms of MM are still poorly understood, which results in complicated therapies [1, 3, 4]. In spite of advanced progress in diagnosis and treatment, including immunomodulatory medicine, proteasome inhibitors and autologous stem cell transplantation, the prognosis of MM is also dismal. As a consequence, it is important to look for therapies that either complement or replace the existing strategies in order to extend the survival of MM patients [5, 6].

Long non-coding RNAs (lncRNAs, >200 nt), play a critical role in biological processes, such as cell proliferation, differentiation, apoptosis, and migration [7, 8]. Various lncRNAs, such as HOTTIP, MEG3, UCA1 and MALAT1, have been demonstrated to be involved in different carcinomas including MM [9]. MicroRNAs are critical members of non-coding RNAs (ncRNAs), which are 22 nucleotides in length. They modulate gene expressions via binding to the 3'-UTR of mRNAs [10]. It has been indicated in a recent study that lncRNAs have targeting relationships with miRNAs, which therefore regulates the expressions of target genes [11]. For instance, knockdown of lincRNA MALAT1 has been found increasing the blood-tumor barrier permeability by up-regulating miR-140, as supported by Ma et al. [12] LncRNAs and miRNAs both play vital roles in several kinds of cancers, but the specific mechanism remains to be discussed. A complete understanding of the function of lncRNAs and miRNAs will contribute to novel therapeutic strategies.

Autophagy is an essential catabolic process of cytoplasmic contents, which is able to assistant cells to maintain homeostasis [8, 13]. Autophagy can be divided into at least three types by their physiological roles and the pattern of cargo delivery to the lysosome: chaperone-mediated autophagy, microautophagy and macroautophagy. Macroautophagy, the main catabolic mechanism, is modulated by a limited number of autophagy related genes (*ATG*). Autophagy and its molecular mechanism in carcinomas are under deep discussion by recent studies, and the role of *ATG14* (Autophagy Related 14) in this process has gained more attentions [14]. *ATG14* is a protein coding gene. Among its related pathways are autophagy pathway and senescence and autophagy in cancer. A recent study has shown that *ATG14*, a crucial member of *ATG* gene family, plays a vital role in autophagy processes, [15] which provides protections to cancer cells from the deadly stresses that result from radiation, chemotherapy or other treatments [16]. However, how cancer cells modulate the expression of *ATG* to prevent cell death caused by radiation and chemotherapy still needs to be discussed.

At early step in autophagy, ATGs (autophagy related genes) work in concert to conjugate phosphatidylethanolamine to LC3-I to form LC3-II. The latter gets recruited to the autophagosomal membrane for helping membrane elongation. ATG7 also mediates ATG5-ATG12-ATG16 complex formation and the latter along with LC3-II is highly critical for autophagosome formation [17, 18]. Adaptor protein p62/SQSTM1 binds to ubiquitinated proteins and LC3-II for mediating autophagy via localizing into autophagic compartments, transporting ubiquitinated proteins and organelles for degradation [19]. Based on these properties of LC3-I, LC3-II and p62, those were used to measure autophagic flux under certain conditions.

Therefore, this study investigated the relative expression of linc00515 in chemoresistant myeloma cells via RT-PCR, and investigated the effects of linc00515 on cell resistance and autophagy. We investigated the regulation mechanism by figuring out the targeting relationship of linc00515, miR-140-5p and *ATG14*. Our study may provide novel ideas on diagnosis and therapy of MM.

Materials and Methods

Patient and clinical features

43 MMs with newly diagnosed IgG MM were collected in the study from patients at the Fourth Affiliated Hospital of Harbin Medical University between June 2015 and Septembre 2017, who ranged in age from 35 to 84 years with a mean of 59.0 years. 14 control cases whose age ranged from 36 to 85 years with a mean age of 60.5 years underwent routine physical examinations. Patients with acute or chronic infection, inflammatory processes and liver or kidney diseases (creatinine above 2.0 mg/dl or creatinine clearance rate CrCl below 60 ml/min) had been excluded from the study. Complete blood count, erythrocyte sedimentation rate, total protein/albumin, protein and immune electrophoresis, renal function tests and skeletal surveys were performed in all cases. In addition, both tumor and normal tissue samples were confirmed by pathological examination. At the time of diagnosis, all patients were divided into three groups based on international staging system, ISS (12 patients in stage I, 11 patients in stage II, and 20 in stage III). According to the Durie-Salmon staging system, 7 patients had stage I disease, 7 patients stage II, and 29 patients stage III. Bone marrow samples were snap-frozen in liquid nitrogen until RNA extraction. No local or systemic treatment had been conducted before operation. The study was approved by the Fourth Affiliated Hospital of Harbin Medical University Ethical Committee, and informed consent was obtained from each patient prior to the study. The detail clinical features of patients were shown in Table 1.

Table 1. Clinical features of the patients. The values are presented as mean ± SD. ISS: international staging system; DS: Durie-Salmon staging system; HGB: haemoglobin; M: monoclonal; Ca: calcium; IgG: immunoglobulin G; LDH: lactate dehydrogenase; TB: trephine biopsy; BM: bone marrow; PLT: platelets counts; WBC: white blood cells

Characteristics	Outcome Measured
Number of patients	43
Age	59 (range 35-84)
Stage ISS	
I	12
II	11
III	20
Stage DS	
I	7
II	7
III	29
Solitary plasmocytoma	0
HGB (g/dl)	9.87 ± 1.66
Serum protein (g/dl)	9.48 ± 1.98
Ca^{2+} (mmol/l)	2.51 ± 0.33
IgG (mg/dl)	6741.0 ± 1294.3
Serum albumin (g/dl)	3.19 ± 1.04
β2 microglobulin (g/l)	9.12 ± 4.57
LDH (IU/l)	237.8 ± 156.01
% plasma cells in TB	43.15 ± 25.42
% plasma cell in smear BM	39.13 ± 22.68
Creatine level (mg/dl)	1.68 ± 0.36
PLT ($\times 10^3$)	234.8 ± 112.8
WBC ($\times 10^3$)	5.98 ± 3.26

Cell line and cell culture

MM cell line LP1 and KMS11 were purchased from BeNa Culture Collection (Shanghai, China), and cultured in 90% RPMI-1640 medium (GIBCO, #31800022) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). To generate melphalan-resistant LP1 and KMS11 cell line, LP1 cells were exposed to melphalan at a concentration that induced 25% proliferation inhibition for one week. The drug dose was then increased fortnightly until the cell line was resistant to 10 μM. LP1 and KMS11 cells were then cultured in drug-free media and their resistance phenotype was confirmed monthly. Melphalan-resistant LP1 cells underwent drug-free culture for at least 2 weeks before experiments. 293T cells were purchased from ATCC and were cultured in DMEM medium with 10% FBS.

Table 2. qRT-PCR Primer Sequences

Primer	Sequence
linc00515 forward	5'-AACGGAGCAGTGATGTGGTT-3'
linc00515 reverse	5'-TGCGGGGGAAAAACTGCTAA-3'
miR-140-5p forward	5'-CAGTGGTTTTACCCTA-3'
miR-140-5p reverse	5′-GTGCAGGGTCCGAGGT-3'
ATG14 forward	5′- AAAGACGGGTGTGAGAGACC-3′
ATG14 reverse	5'- GGTGTCTCCGTTGTGATCGT-3'
GAPDH forward	5'-GCGACACCCACTCCTCCAC-3'
GAPDH reverse	5′-TCCACCACCCTGTTGCTGTAG-3′
U6 forward	5′-CTCGCTTCGGCAGCACA-3′
U6 reverse	5′-AACGCTTCACGAATTTGCGT-3′

RT-PCR assay

Total RNA was extracted by TRIzol reagent (Invitrogen). After determining the quantity with NanoDrop 2000 (Thermo Fisher Scientific Inc, USA), 2.0 ng of total RNA was used for reverse transcription (RT) using ReverTra Ace qPCR RT Kit (Toyobo, Japan). PCR was performed as follows: 2 min initial denaturation at 94°C; 30 s denaturation at 94°C; 30 s annealing at 56°C; 1 min extension at 72°C, 30 cycles and 10 min extension at 72°C. The procedure was repeated in each group for three times. Relative RNA expression levels were expressed as $2^{-\Delta\Delta CT}$. The primers sequences for RT and real-time PCR are shown in Table 2.

Western blot assay

Cells were lysed using RAPI (Beyotime, Shanghai, China) and 100 μg of protein samples were analyzed using SDS-polyacrylamide gel electrophoresis followed by semi-dry transfer onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated in TBST containing 5% nonfat milk for 1 h. Proteins in membrane were experienced incubation with anti-*ATG14* (ab80261, 1 μg/ml, Abcam, Cambridge, MA, USA), anti-p62 (ab91526, 1:1000, Abcam), anti-LC3B (ab48394, 1 μg/ml, Abcam) and anti-β-actin (ab8227, 1:2000, Abcam) at 4°C overnight. The membrane was washed by TBST for 3 times and experienced incubation with goat anti-mouse IgG (HRP) (ab205718, 1:2000, Abcam) at room temperature for 1.5 h. After 3 times TBST washing, the membrane was subjected to color reaction by ECL Plus from Life Technology, and β-actin was used as the internal control.

Cell transfection

MiR-140-5p mimics (MH10205), anti-miR-140-5p inhibitor (MH10205) were purchased from Thermo Fisher. The sequences of si-linc00515 (GGGCTCACCAATTAGACAT) and si-*ATG14* (GCGGCGATTTCGTCTACTT) were designed by Thermo Fisher and synthesized by GenePharma Technology Co., Ltd. All cells were maintained media containing 6 μM/mL melphalan. Lipofectamine 3000 (Life Technologies, USA) was used as the transfection reagent. All cells were collected 48 hours after transfection for further experiments.

MTT assay

Cells were seeded at a density of 1, 000-10, 000 cells/well in 96-well plates in 200 μl cell culture medium. After the cells were cultured at a temperature of 37°C for 3-5days, 10 μl MTT was added to each well at a final concentration of 5 mg/ml, and the cells were incubated for another 4 h. Then the medium was removed, and the precipitated formazan was dissolved in 100 μl DMSO. Cell viability was assessed by measuring the absorbance of each well on an enzyme-linked immunosorbent plate reader.

Flow cytometry assay

A cell apoptosis assay was performed using flow cytometry with propidium iodide (PI) and AnnexinV-FITC double staining. 24 h after transfection, cells were collected, washed and resuspended. AnnexinV-FITC and PI were used for double staining to assess the proportions of apoptotic cells. Data were read with FACScan flow cytometer (Cytekdev, USA)

Dual-luciferase reporter gene assay

To construct dual luciferase reporter plasmids, wild-type (WT) and mutated (MUT) 3'-UTR of linc00515 and *ATG14* were generated by PCR amplification. The amplified sequences were connected with Pmir-GLO Dual-Luciferase miRNA Target Expression Vectors (Promega, Madison, WI, USA). 293T cells were seeded onto 24-well plates for plasmid co-transfection with constructed miR-140-5p mimics or negative control (NC) mimics. Firefly luciferase activity and Renilla luciferase activity were both measured 48 h later as instructed by the Dual-Luciferase Reporter Assay System protocol, and the measure of cell relative luciferase activity was calculated as their ratio.

GFP-LC3 fluorescence microscopy

Melphalan-resistant and melphalan-sensitive LP1 cells were inoculated in 24-well plates and transfected with si-linc00515, si-ATG14, si-linc00515+miR-140-5p inhibitor or si-ATG14+ miR-140-5p inhibitor using Lipofectamine 3000. Afterwards, 200 ml of the complexes were added to 24-well plates and mixed gently. Incubation was subsequently conducted, followed by media replacement after 4 h cell culture. 48 h post-transfection, cells were treated with 1 μM melphalan for 2 h prior to fixation. Nuclei were stained

with DAPI and LC3 was stained with GFP-LC3 (ab48394, Abcam). Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan) was adopted to observe the number of green dots in cells on anti-quenched slides in the well plate. Generally, if there were more than 5 green highlights in a cell, it was identified as an autophagy-positive cell, with random 4 horizons per well and at least 3 wells for each group.

Statistical analysis

The data in this study were expressed as the mean ± SD. Two groups were compared with Student's t-test. Multiple groups were compared with One-Way ANOVA. Statistical analysis was completed with Graphpad Prism 6.0 software. Significance level was set as $P<0.05$.

Results

Linc00515 was overexpressed in melphalan-resistant and under-expressed in sensitive LP1 cells

In order to explore the role of linc00515 in MM, We firstly detected the expression of linc00515 in MM and normal samples, as well as LP1, KMS11 and HS-27A cells. Linc00515 was overexpressed in MM samples and MM cells (LP1 and KMS11) compared with normal samples and human bone marrow cells (HS-27A) (Fig. 1A&B). To confirm the successful

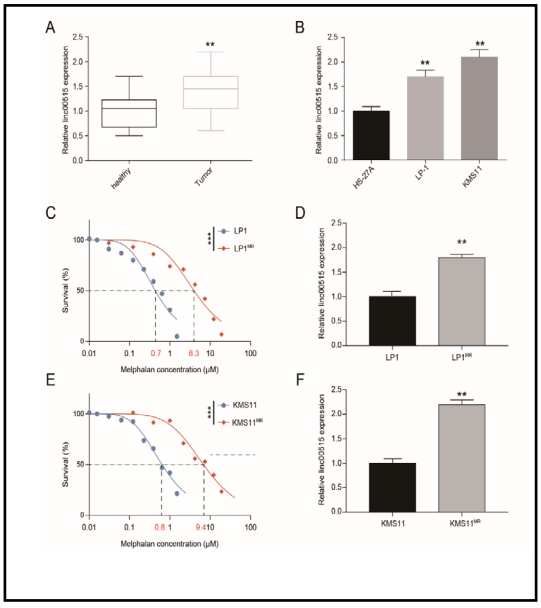

Fig. 1. Linc00515 was overexpressed in melphalan-resistant and under-expressed in sensitive MM cells. A Linc00515 was overexpressed in MM samples. B The expression of linc00515 in LP1, KMS11 and HS-27A cells were detected by Q-PCR. ** P<0.01, compared with HS-27A cells. C The IC50 value for LP1 and LP1^{MR} after treated melphalan. D Linc00515 was upregulated in LP1^{MR} cells. ** P<0.01, compared with LP1 cells. E The IC50 value for KMS11 and KMS11^{MR} after treated melphalan. F Linc00515 was upregulated in KMS11^{MR} cells. ** P<0.01, compared with KMS11 cells.

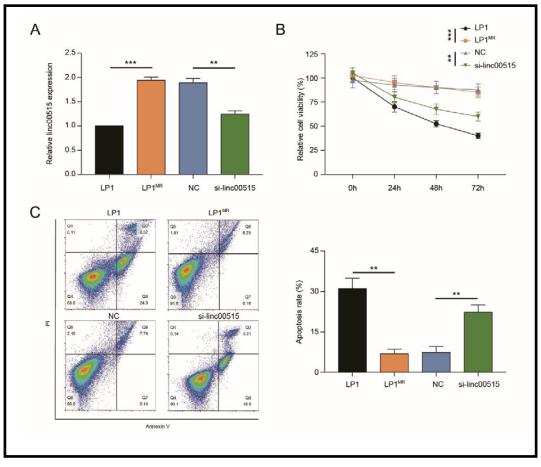

Fig. 2. Knockdown of linc00515 reduced the melphalan-resistance of LP1^{MR} cells. A The knockdown of linc00515 significantly suppressed the expression of linc00515 in LP1^{MR} cells. ** P<0.01, *** P<0.001. B The knockdown of linc00515 significantly compromised cell viability of LP1^{MR} cells treated with melphalan. ** P<0.01, compared with NC group. C The knockdown of linc00515 significantly promoted cell apoptosis of LP1^{MR} cells treated with melphalan. ** P<0.01, compared with NC group.

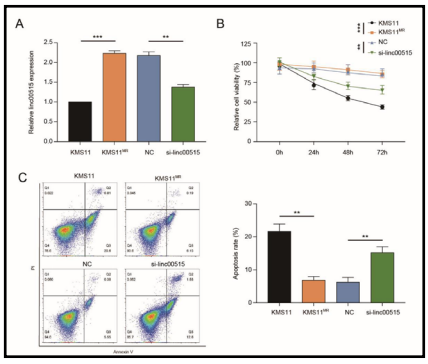

Fig. 3. Knockdown of linc00515 reduced the melphalan-resistance of KMS11^{MR} cells. A The knockdown of linc00515 significantly suppressed the expression of linc00515 in KMS11^{MR} cells. ** P<0.01, *** P<0.001. B The knockdown of linc00515 significantly compromised cell viability of KMS11^{MR} cells treated with melphalan. ** P<0.01, compared with NC group. C The knockdown of linc00515 significantly promoted cell apoptosis of KMS11^{MR} cells treated with melphalan. ** P<0.01, compared with NC group.

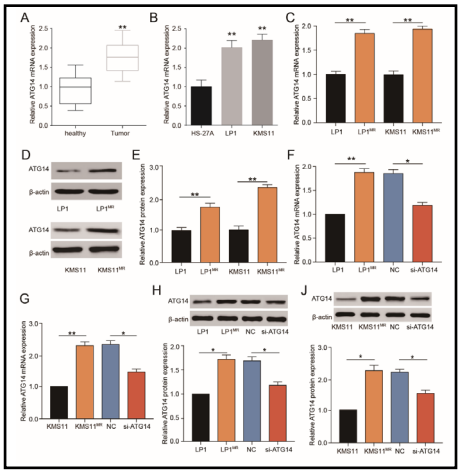

Fig. 4. ATG14 was up-regulated in melphalan-resistant MM cells. A ATG14 mRNA was shown to be overexpressed in MM samples. B The mRNA expression of ATG14 in LP1, KMS11 and HS-27A cells were detected by Q-PCR. ** P<0.01, compared with HS-27A cells. C The mRNA of ATG14 was upregulated in LP1^{MR} and KMS11^{MR} cells. ** P<0.01, compared with LP1 cells or KMS11 cells. D-E The protein of ATG14 was overexpressed in LP1^{MR} and KMS11^{MR} cells. ** P<0.01, compared with LP1 cells or KMS11 cells. F-J ATG14 knockdown cell line was successfully constructed by si-ATG14 transfection tested at mRNA and protein level in LP1^{MR} and KMS11^{MR} cells. * P<0.05, compared with NC group;

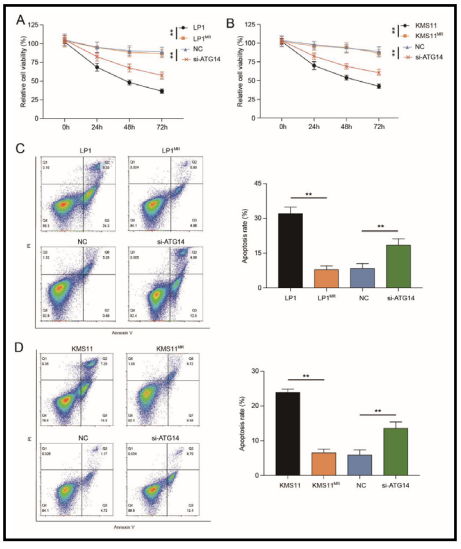

Fig. 5. Knockdown of ATG14 reduced the melphalan-resistance of myeloma cells. A The knockdown of ATG14 significantly inhibited the viability of LP1^{MR} cells treated with melphalan. ** P<0.01, compared with NC group or LP1 group. B The down-expression of ATG14 significantly inhibited the viability of KMS11^{MR} cells treated with melphalan. ** P<0.01, compared with NC group or LP1 group. C Down-regulated ATG14 significantly promoted the apoptosis of LP1^{MR} cells treated with melphalan. ** P<0.01, compared with NC group or LP1 group. D The knockdown of ATG14 observably promoted the apoptosis of KMS11^{MR} cells treated with melphalan. ** P<0.01, compared with NC group or LP1 group.

construction of melphalan-resistant LP1 and KMS11 cell line, we measured the IC50 of both sensitive (LP1 and KMS11) and resistant (LP1$^{MR\ (melphalan\ resistant)}$ and KMS11^{MR}) MM cells. The results showed that the IC50 for LP1 and LP1^{MR} were 0.659±0.92 μM and 7.795±2.34 μM, and the IC50 for KMS11 and KMS11^{MR} were 0.835±0.76 μM and 9.417±3.12 μM, respectively, indicating the successful construction of LP1^{MR} and KMS11^{MR} cell line (Fig. 1C&E). The relative expression of linc00515 was measured in sensitive (LP1 and KMS11) and resistant (LP1^{MR} and KMS11^{MR}) MM cells that were cultured in media without melphalan. The results showed that LP1^{MR} and KMS11^{MR} had significantly higher lnc00515 level (P<0.01, Fig. 1D&F).

Knockdown of linc00515 reduced the melphalan-resistance of myeloma cells

The knockdown of linc00515 significantly suppressed the expression of linc00515 in LP1^{MR} cells (P<0.01, Fig. 2A). Cell viability was compromised, and cell apoptosis was promoted in LP1^{MR} cells treated with melphalan as a result of the knockdown of linc00515 (P<0.01, Fig. 2B&C). The similar results were presented in KMS11^{MR} cells (P<0.01, Fig. 3). In short, down-expression of linc00515 reduced the melphalan-resistance of myeloma cells.

Knockdown of ATG14 reduced the melphalan-resistance of myeloma cells

ATG14 was upregulated in MM samples and MM cells (LP1 and KMS11) compared with normal samples or human bone marrow cells (HS-27A) (Fig. 4A&B). To further explore the relationship of *ATG14* and melphalan-resistance of myeloma cells, we detected the mRNA and protein levels of *ATG14* by Q-PCR and western blot. *ATG14* was shown to be overexpressed in LP1^{MR} and KMS11^{MR} cells at both transcriptional and translational levels (P<0.01, Fig. 4C-E). *ATG14* knockdown cell line was successfully constructed by si-ATG14 transfection (P<0.05, Fig. 4F-J). The knockdown of *ATG14* significantly contributed to the inhibition of the viability and the promotion of the apoptosis of LP1^{MR} and KMS11^{MR} cells treated with melphalan (P<0.01, Fig. 5A-D).

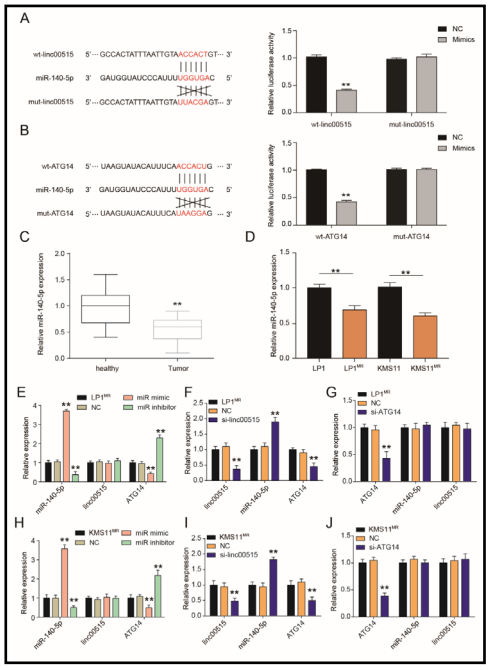

Fig. 6. Linc00515 downregulated the expression of ATG14 by sponging miR-140-5p. A Bioinformatics analysis predicted that linc00515 was a potential target of miR-140-5p and luciferase report assay of linc00515-luc and it's mutant treated with miR-140-5p mimic and NC. ** P<0.01, compared with NC group. B Bioinformatics analysis predicted that ATG14 3' UTR was a potential target of miR-140-5p and luciferase report assay of ATG14-luc and it's mutant treated with miR-140-5p mimic and NC. ** P<0.01, compared with NC group. C miR-140-5p was down-expressed in MM samples. D miR-140-5p expression in LP1, LP1^{MR} KMS11 and KMS11^{MR} cells was detected by Q-PCR. ** P<0.01, compared with LP1 or KMS11 cells. E&H The expression of miR-140-5p, linc00515 and ATG14 in LP1^{MR} and KMS11^{MR} transfected with miR-140-5p mimic, inhibitor and NC. ** P<0.01, compared with NC group. F&I The expression of linc00515, miR-140-5p and ATG14 in LP1^{MR} and KMS11^{MR} after transfected with si-linc00515 and NC. ** P<0.01, compared with NC group. G&J The expression of ATG14, miR-140-5p and linc00515 in LP1^{MR} and KMS11^{MR} transfected with si-ATG14 and NC. ** P<0.01, compared with NC group.

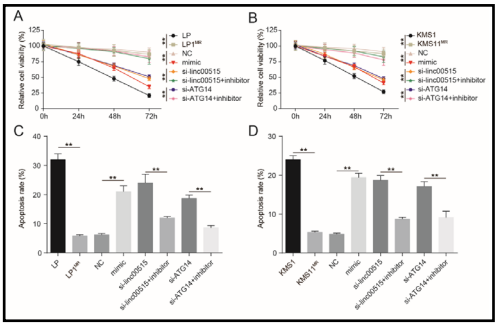

Fig. 7. The effects of linc00515/miR-140-5p/ATG14 on the viability and apoptosis LP1^{MR} and KMS11^{MR} cells. A&B The relative cell viability of LP1^{MR} and KMS11^{MR} transfected with miR-140-5p mimic, si-linc00515, si-ATG14, si-linc00515 plus miR-140-5p inhibitor, si-ATG14 plus miR-140-5p inhibitor and NC co-treated with melphalan. ** P<0.01. C&D The apoptosis of LP1^{MR} and KMS11^{MR} transfected with miR-140-5p mimic, si-linc00515, si-ATG14, si-linc00515 plus miR-140-5p inhibitor, si-ATG14 plus miR-140-5p inhibitor and NC co-treated with melphalan. ** P<0.01.

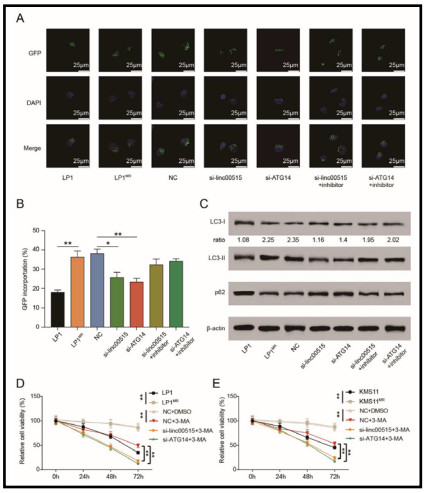

Fig. 8. Linc00515, ATG14 and miR-140-5p inhibitor promoted the autophagy of myeloma cells. A&B GFP-LC3 fluorescent analysis in LP1^{MR} after transfected with si-linc00515, si-ATG14, si-linc00515 plus miR-140-5p inhibitor, si-ATG14 plus miR-140-5p and the results indicated that the knockdown of linc00515 and ATG14 inhibited the autophagy of melphalan-resistance myeloma cells whereas miR-140-5p inhibitor compromised this effect. *P<0.05, compared with NC group; **P<0.01, compared with NC or LP1 group. C Western blot assay showed the LC3 I, LC3 II and p62 protein expression in LP1^{MR} after transfected with si-linc00515, si-ATG14, si-linc00515 plus miR-140-5p inhibitor, si-ATG14 plus miR-140-5p. D&E The relative cell viability of LP1^{MR} and KMS11^{MR} transfected with si-linc00515, si-ATG14, NC co-treated with 3-MA. All groups were treated melphalan. **P<0.01.

The effects of linc00515/miR-140-5p/ATG14 on the viability and apoptosis LP1^{MR} cells

The binding sequences of linc00515, miR-140-5p and *ATG14* were illustrated. Also, we gave the mutated binding sequences of linc00515 and *ATG14*. Results were shown by the dual-luciferase reporter gene assay that linc00515 directly targeted miR-140-5p, which directly bound to *ATG14* (Fig. 6A-B). miR-140-5p was found down-regulated in MM samples (P<0.01, Fig. 6C). Besides, miR-140-5p was found downexpressed in LP1^{MR} cells (P<0.01, Fig. 6D). The mimics and inhibitor of miR-140-5p significantly modulated the expression of miR-140-5p in LP1^{MR} and KMS11^{MR} cells (P<0.01, Fig. 6E&H). The transfection of miR-140-5p mimics and inhibitor observably altered the expression of *ATG14*, but not the linc00515 levels (P<0.01, Fig. 6E&H). Down-expression of linc00515 significantly promoted miR-140-5p expression and downregulated *ATG14* expression (P<0.01, Fig. 6F&I). In addition, down-expression of *ATG14* by si-ATG14 had no influence on the expression of linc00515 and miR-140-5p (Fig. 6G&J). Those results suggested that linc00515 could sponge miR-140-5p directly and then promote expression of *ATG14*. The transfection of miR-140-5p mimics significantly suppressed cell viability but promoted cell apoptosis. The simultaneous suppression of miR-140-5p and linc00515 or *ATG14* reversed the inhibition of cell viability and the facilitation of cell apoptosis induced by si-linc00515 or si-*ATG14* alone (P<0.01, Fig. 7A-D).

Linc00515, ATG14 and miR-140-5p inhibitor promoted the autophagy of myeloma cells

The results of GFP-LC3 fluorescent analysis indicated that the knockdown of linc00515 and *ATG14* inhibited the autophagy of myeloma cells whereas miR-140-5p inhibitor compromised this effect (Fig. 8A-B). Western blot assay showed that the LC3 II/LC3 I ratio was much lower in si-linc00515 and si-ATG14 groups, compared with NC group. The simultaneous suppression of miR-140-5p and linc00515 or *ATG14* led no not significantly different of LC3 II/LC3 I ratio compared with NC group (Fig. 8C). The expression of p62 was much higher in si-linc00515 and si-ATG14 groups compared with NC group, while co-transfected miR-140-5p inhibitor reversed the expression of p62 (Fig. 8C). To explore the role of autophagy in melphalan-resistance of myeloma cells, an inhibitor of autophagy, 3-Methyladenine (3-MA, Selleck Chemicals, Houston, TX, USA), was selected. The sensitive of LP1^{MR} and KMS11^{MR} to melphalan were enhanced after 3-MA treatment (5 mM). Besides, down-expression of linc00515 or *ATG14* strengthen the inhibition of 3-MA, compared with 3-MA treatment alone (Fig. 8D-E).

Discussion

In this study, we demonstrated that linc00515 was overexpressed in melphalan-resistant and under-expressed in sensitive LP1 and KMS11 cells. Knockdown of linc00515 and *ATG14* reduced the melphalan-resistance of myeloma cells. Linc00515 directly targeted miR-140-5p, which directly bound to *ATG14.* The transfection of miR-140-5p mimics significantly suppressed cell viability but promoted cell apoptosis. Linc00515, *ATG14* and miR-140-5p inhibitor promoted the autophagy of myeloma cells.

Previous studies have verified that the expression level of several lincRNAs was higher in MM cells than in normal ones. For instance, Sui et al. observed the expression of lincRNA MALAT1, which was overexpressed in MM patients [20]. The expression of lincRNA PCAT1 was found to be higher in MM group than in healthy control groups by Shen et al. [6]. However, there has been no report on the expression of linc00515 in drug-resistant cell lines. Intriguingly, the expression of linc00515 was detected to be overexpressed in LP1 melphalan resistant cells in our study, indicating that linc00515 not only promoted carcinogenesis but also enhanced the drug resistance of MM cells.

Our study also corroborated that knockdown of linc00515 and *ATG14* reduced the melphalan-resistance of myeloma cells, which could be confirmed by related findings about lincRNA and *ATG14* involved in tumorigenesis. Knockdown of *ATG14* was indicated by He et al. to promote apoptosis of ovarian cancer cells. For further certification, the effect of ATG7 on resistance was tested as well. Similarly, ATG7 deficiency promoted apoptosis of human esophageal squamous cell carcinoma cells [16]. Inhibition of ATG5 enhanced cisplatin induced apoptosis and decreased cell viability according to a recent study by Ma et al. [8] Zhao et al. suggested that silencing of *ATG14* caused higher apoptosis rates in sensitizing osteosarcoma cells to cisplatin [13]. These results indicate that *ATG14* is capable of promoting chemoresistance of cancer cells. As for linc00515 involvement in tumor cell apoptosis, there was no direct evidence from previous researches. Our study showed that MM cell apoptosis could be promoted by linc00515 overexpression, which was a supplement to the hypothesis on its role as a tumor suppressor.

Previous studies suggested that *ATG14* regulated autophagy in several cell types, but its role in MM cells was still unknown. Present results showed that the knockdown of linc00515 and *ATG14* inhibited the autophagy of myeloma cells whereas miR-140-5p inhibitor promoted it. He et al. reported that the autophagy was reduced in *ATG14* knockdown ovarian cancer cells, which confirmed the contribution of *ATG14* to autophagy in tumor cells [16]. As for roles of lincRNAs in MM autophagy, there are few studies currently. However, a recent study by Yuan et al. indicated that lincRNA MALAT1 inhibition and miR-216b suppressed autophagy of hepatocellular carcinoma cells, [21] and Li et al. reported that the inhibition of lncRNA ROR increased the expressions of LC3 and Beclin 1 [22]. These results showed that several kinds of lincRNAs promoted autophagy in cancer cells, which verified our results indirectly.

Overall, investigating how lnc00515, miR-140-5p and *ATG14* participated in MM procedure was a great attempt for us. However, limitations still existed in this study. For example, we successfully investigated the relationship between linc00515, miR-140-5p and *ATG14* and their effects on MM cell autophagy and melphalan resistance, but the detailed regulation mechanism between linc00515 and miR-140-5p in MM cells was still unclear and future experiments should prove our present conclusion.

In conclusion, linc00515 contributed to the up-regulation of *ATG14* in myeloma cells via inhibiting the expression of miR-140-5p. Linc00515 directly targeted miR-140-5p, which directly bound to *ATG14*. Up-regulation of *ATG14* promoted autophagy of myeloma cells, which further promoted myeloma chemoresistance to melphalan. In conclusion, knockdown of linc00515 inhibited multiple myeloma autophagy and chemoresistance by up-regulating miR-140-5p and down-regulating *ATG14.*

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Disclosure Statement

The authors declare that they have no potential conflicts of interest.

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