Tohoku J. Exp. Med., 2025, **265**, 99-111

The Molecular Mechanism by which LINC00461 Regulates Carfilzomib Resistance in Multiple Myeloma

Lifang Cheng[1] and Fanjuan Zhang[2]

[1]Department of Haematology, Shenshan Medical Center, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Shanwei, Guangdong, China
[2]Department of Emergency, Shenzhen Samii Medical Center, Shenzhen, Guangdong, China

Multiple myeloma (MM) is a frequent haematological malignancy of the bone marrow. Carfilzomib, a first-line treatment for MM, has excellent antitumour effects, but its efficacy eventually decreases due to primary or acquired chemoresistance. Therefore, the regulatory mechanism of carfilzomib resistance has attracted much attention for improving the survival outcomes of patients with MM. By using database analysis combined with quantitative real-time polymerase chain reaction (qRT–PCR), aberrant lncRNAs in MM were screened in carfilzomib-resistant cells versus carfilzomib-sensitive cells, and the resistance index of cultured carfilzomib-resistant cells was analysed compared to that of parental cells. Furthermore, cell viability, proliferation, and apoptosis in response to treatment with carfilzomib were measured after treatment with LINC00461 by the cell counting kit-8 (CCK-8) method and flow cytometry. The expression of LINC00461 was also verified through qRT–PCR. Then, the possible miRNA molecules on which LINC00461 may act were investigated by RNA immunoprecipitation (RIP) and dual-luciferase reporter assays. Next, tumorigenesis in mice was evaluated to verify the effect of LINC00461 on carfilzomib-resistant cells. Increased expression of LINC00461 was related to drug resistance in MM patients. Mechanistically, LINC00461 overexpression attenuated the effect of miR-539-3p overexpression and decreased the expression of the downstream protein RAB5A. Moreover, compared with the control group, the LINC00461 knockdown group treated with carfilzomib exhibited decreases in tumour volume and weight. Furthermore, LINC00461 sensitized carfilzomib-sensitive cells by promoting the release of exosomes. These data suggest that LINC00461 plays an important role in the development of carfilzomib resistance.

Keywords: carfilzomib resistance; LINC00461; miR-539-3p; multiple myeloma; RAB5A
Tohoku J. Exp. Med., 2025 February, **265** (2), 99-111.
doi: 10.1620/tjem.2024.J082

Introduction

Multiple myeloma (MM) is the second most common malignant tumour among blood system cancers (Anderson 2004), with more than 20,000 newly diagnosed cases every year. At present, treatment based on proteasome inhibitors is the cornerstone of MM therapy. Although great progress has been made in the prognosis of MM, relapse activity due to drug resistance is still a major clinical challenge (Abdi et al. 2013).

Carfilzomib is a highly effective second-generation proteasome inhibitor that can play an antitumour role by inducing the apoptosis of MM cells in vitro. A preclinical study, revealed that reducing ABCB1 activity could overcome carfilzomib resistance in MM (Besse et al. 2018). Riz et al. (2015) reported that autophagy is involved in the carfilzomib resistance of MM to carfilzomib, and the author found that inhibiting the mTOR pathway increased the sensitivity of carfilzomib-resistant MM cells to carfilzomib by regulating miRNA expression in MM (Acosta-Alvear et al. 2015). In this study, we first revealed that LINC00461 confers drug resistance, and then the detailed molecular mechanism of carfilzomib resistance in MM was elucidated.

Long noncoding RNAs are a set of transcripts with a length of more than 200 nucleotides that have extensive biological effects at multiple levels. An increasing number of studies have shown that the abnormal expression of lncRNAs is closely related to tumour cell growth, apopto-

Received May 14, 2024; revised and accepted August 25, 2024; J-STAGE Advance online publication September 5, 2024
Correspondence: Fanjuan Zhang, Department of Emergency, Shenzhen Samii Medical Center, Shenzhen Guangdong 518118, China.
e-mail: goodfanjuan@163.com

sis, metastasis, and chemotherapy resistance (Ma et al. 2020). LINC00461 inhibits the progression of rectal cancer, promotes cisplatin sensitivity in rectal cancer cells (Qu et al. 2020), and can promote docetaxel resistance in breast cancer (Zhang et al. 2020). In addition, knocking down LINC00461 inhibits the proliferation, migration, invasion and temozolomide resistance of glioma cells (Peng et al. 2020). LINC00461 can also regulate sunitinib resistance in renal cell carcinoma through miR-942 (Chen et al. 2019). Therefore, targeting oncogenic LINC00461 may be a prospective approach for restoring chemosensitivity in MM. In addition, lncRNAs can affect protein expression by targeting miRNAs. Previous reports have shown that miR-324-3p, miR-15a and miR-16 are downstream targets of LINC00461. In our work, the results showed that miR-539-3p is linked to the functional activity of lncRNAs. In our study, RAB5A, which is involved in material transduction, receptor regulation, and cytoskeleton reconstruction in cancer (Li et al. 2017), was found to be a direct target gene of miR-539-3p by dual luciferase reporter assay. However, there are few reports on the role of miR-539-3p and RAB5A in carfilzomib resistance. Overall, the associations among LINC00416, miR-539-3p, and RAB5A in modulating carfilzomib resistance require further research.

In this study, we hypothesized that LINC00416 could regulate RAB5A through a miR-539-3p sponging effect. Herein, loss-of-function assays indicated that LINC00461 knockdown induced decreased proliferation while increasing more apoptosis in carfilzomib-resistant cells in vitro. Importantly, in vivo experiments revealed that the tumour growth can be delayed by LINC00461 knockdown combined with carfilzomib treatment. Moreover, the high expression of LINC00461 promoted the release of exosomes, and ultimately facilitated the occurrence of carfilzomib resistance in MM.

Materials and Methods

Quantitative real-time polymerase chain reaction (qRT–PCR)

The cells were washed and lysed, and total DNA was extracted. Then, the amount of normalized double-stranded DNA was measured on a 384-well qRT–PCR plate (ABI 7500; Applied Biosystems, Waltham, MA, United States). After the absorbance values of the samples were detected at 260 nm and 280 nm, reverse transcription experiments were conducted to obtain cDNA. The data were processed using the $2^{-\Delta\Delta Ct}$ quantification method, and the relative expression levels of the target genes were obtained using GAPDH or U6 as the internal control. The experimental cycles were as follows: 95°C for 5 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 10 min. The primer sequences are listed in Supplementary Fig. S1.

Construction of lentiviruses

Human KMS11 or KMS34 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka Japan), KMS11-CR or KMS34-CR cells were induced by increased carfilzomib concentration and survived at the final concentration of 92 nM or 105 nM respectively. Lentiviruses carrying the LINC00461 regulation vector or empty vector were constructed by Synbio Tech (Suzhou, China). The target sequences of the shRNAs were as follows: shLINC00461-1 5′-CTGCAAAGAAGCATAAAATGA-3′ and shLINC00461-2 5′-AATTCTCCGAACGTGTCACGT-3′. The process was as follows: cell transfection, virus harvesting, sample concentration and transformation strain acquisition. Mock-transduced cells were used as the negative control, and qRT–PCR was used to confirm the efficiency of the derived virus.

Cell counting kit-8 (CCK-8) assay

The CCK-8 method was used to detect drug sensitivity in MM cells. The cells were diluted and transferred to 96-well plates. Ten microlitres of a commercial CCK-8 kit (Dojindo, Molecular Technologies, Kumamoto, Japan) was added to every plate after the cells were treated with carfilzomib at a dose of 1, 10, 20, 30, 60, or 90 nm. The absorbance value of each well was measured at a wavelength of 450 nm. The 50% inhibitory concentration (IC_{50}) values for KMS11 and KMS11-CR cells were 8.2 and 95 nm respectively, as calculated by SPSS 19.0 software.

Western blot experiment

Cells with good growth status were lysed with a protease inhibitor cocktail. The extracted protein samples were transferred onto the polyvinylidene fluoride membranes. Then, the membranes were sequentially incubated with primary antibodies and HRP-conjugated secondary antibodies. The antibodies used were as follows: RAB5A (cat_not_ab66746), c-Jun (cat_not_ab40766), CD9 (cat_not_ab236630), HSP70 (cat_not_ab203085), CD63 (cat_not_ab134045) and GAPDH (cat_not_ab8245) (Abcam, Cambridge, UK). After the membranes were finally detected by the Bio-Rad Image Lab system (GEL DOC EZ IMAGER, Bio-Rad, CA, USA), each targeted protein band was analysed by the ImageJ software. Glyceraldehyde phosphate dehydrogenase was used as a loading control.

Flow cytometry assay

Adherent cells were treated with carfilzomib after the addition of trypsin and complete medium to assess the cell apoptosis rate. Next, 10 μl of Annexin V-FITC and propidium iodide (E-CK-A217, Elabscience) were simultaneously added to the cell suspension.

The stage of the cell cycle was determined using a Cell Cycle Staining Kit (MultiSciences, Hangzhou, China). A FACS Cantoll flow cytometer (FACScan; BD Biosciences) was used to analyse the samples, and the data were recorded in triplicate for each condition.

Immunofluorescence staining

The samples were washed and fixed with 4% parafor-

maldehyde for 30 minutes, and 10% goat serum was used to block antigens at room temperature for 1 hour after the cell membrane was disrupted. The cells were incubated with specific antibodies overnight before treatment with secondary antibodies, and DAPI was used to stain the nuclei. The images were collected and saved under a Nikon A1Si laser scanning confocal microscope (Nikon Instruments Inc., Japan).

Dual-luciferase reporter assay

The target fragment was inserted into a luciferase-expressing gene expressing vector. Then the gene-containing and internal reference plasmids were cotransfected into cells using Lipofectamine 2000 (Invitrogen), and the cells were subsequently transfected with the miR-539-5p mimic or miR-NC. Stable clones were screened out. Firefly and Renilla luciferase activity was measured using the commercial Dual-Luciferase Reporter Assay System (Promega Biotech Co., Ltd.).

RNA immunoprecipitation (RIP) experiment

A Magna RIP™ RNA Binding Protein Immunoprecipitation Kit (Millipore, MA, USA) was used to verify the binding relationship between LINC00461 and miR-539-5p. First, the cells were lysed in RIP lysis buffer, and mixed with magnetic beads coated with Ago2 or immunoglobulin G (cat_not_ab172730, Abcam). Immunoprecipitated RNA of LINC00461 and miR-539-5p was measured by qRT–PCR after washing with PBS.

Exosome isolation and uptake assay

The cells were cultured in exosome-free FBS for 2 days. Then, the exosomes derived from centrifugation and filtration were fixed with glutaraldehyde, plated on a copper grid, and stained with phosphotungstic acid solution at room temperature. Finally, the dried copper grids were observed using a Tecnai T20 transmission electron microscope. For the uptake of exosomes, 2 μg of exosomes labelled with PKH67 were cocultured with 1×10^6/mL MM cells for 24 h. After staining the nuclei and resuspending in PBS, the cells were observed under a confocal laser scanning microscope.

Animal experiment

The use of clinical specimens in this study was approved by the Samii Hospital ethics committee, and a total of 16 female mice were raised in separate cages under a 12 h day/night cycle. Four-week-old female nude mice aged 4 weeks were injected with 1×10^7 cells in the subcutaneous area. Animals were randomized into four groups, namely, the KMS11-CR-NC, shLINC00461, and shLINC00461+miR-539-3p inhibitor groups, which received carfilzomib at a dose of 10 mg/kg twice a week. Mouse body weight and tumour size were measured daily, and tumour dimensions were calculated as length × width^2 × 0.5. On the 24th day after cell injection, the mice were sacrificed. and no mice succumbed. The tumour mass was removed for imaging and further study.

Immunohistochemistry analysis

The mouse tumour slides were fixed and dehydrated with ethanol and placed in pure xylene and soft paraffin. Then, the collected wax blocks were cut into slices and baked for 1 hour. The slides were incubated with 3% H_2O_2 and sheep serum after dewaxing and antigen retrieval. Thereafter, the sections were incubated with the primary antibody and second antibody, and DAB and haematoxylin were applied for staining. Finally, the images were captured by microscopy and analysed according to the IHC score.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism software (version 9.0, GraphPad Software Inc., San Diego, CA, US). The data are presented as the mean ± standard deviation and were analysed by Student's t test or one-way ANOVA. The Kaplan-Meier method was applied to assess overall survival followed by the log-rank test. A P value less than 0.05 indicated a significant difference, and all experiments were performed in triplicate.

Results

High expression of LINC00461 in carfilzomib-resistant MM cells

As shown in Fig. 1A, there were notable differences in 18 lncRNAs between carfilzomib-resistant and carfilzomib-sensitive patients from TCGA database, whose data were downloaded from the UCSC Xena (http://xena.ucsc.edu/), and a P value < 0.05 and absolute fold change > 2 were considered the thresholds. Among them, significant differences in H19, LINC00461, and HOXA-AS2 expression were measured by qRT–PCR (Fig. 1B). Next, we measured the expression of LINC00461, HOXA-AS2, and H19 in MM cell lines and found that LINC00461 expression was greater in the more carfilzomib-resistant lines than in the more sensitive lines in MM (Fig. 1C). Thus, LINC00461 was selected as the best candidate in this study. LINC00461 expression was also greater in MM tissues from the databases (Gene Expression Omnibus GSE5900 and GSE2658) than in normal tissues as shown in Fig. 1D. In addition, lncRNAs have widely varying subcellular distributions. Our analysis of LINC00461 localization suggested that LINC00461 was localized mostly to the cytoplasm, revealing its posttranscriptional regulation (Fig. 1E).

LINC00461 facilitates carfilzomib resistance

To study the effect of LINC00461 on the drug resistance of MM, we transfected MM cells with LINC00461 expression vector or empty vector, the results showed that overexpression of LINC00461 in KMS34 cells enhanced carfilzomib resistance by 3.9 times, compared to control cells (Fig. 2A). The CCK-8 assay results showed that the

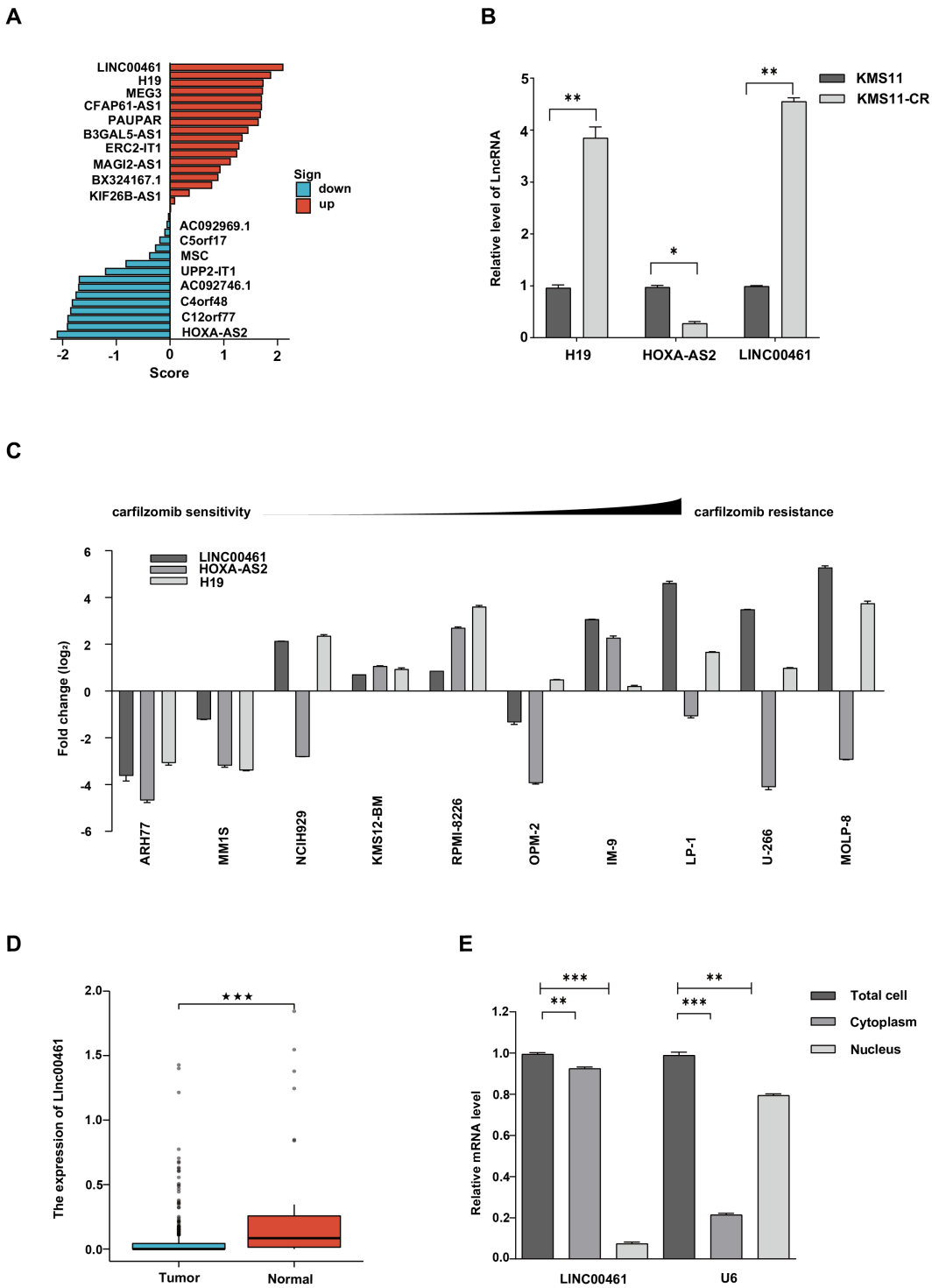

Fig. 1. High expression of LINC00461.
(A) LncRNAs with significantly differential expression between carfilzomib-resistant cells and parental cells. (B) qRT-PCR was used to compare the expression of H19, HOXA-AS2, and LINC00461 between the carfilzomib-resistant group and th control group. (C) qRT-PCR assay was used to detect the expression of H19, HOXA-AS2, and LINC00461 in a panel of 10 cell lines. (D) Differences in the expression of LINC00461 between MM patients and normal controls. (E) Cytoplasmic distribution of LINC00461 in MM cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

IC_{50} value were 5.0 times lower in shLINC00461 cells than control cells (Fig. 2B). As shown in Fig. 2C, the Western blot results indicated that the expression of the apoptotic proteins Bax and Bak was lower in LINC00461-overexpressing cells than in control cells. Consistently, increased expression of Bax and Bak was detected in

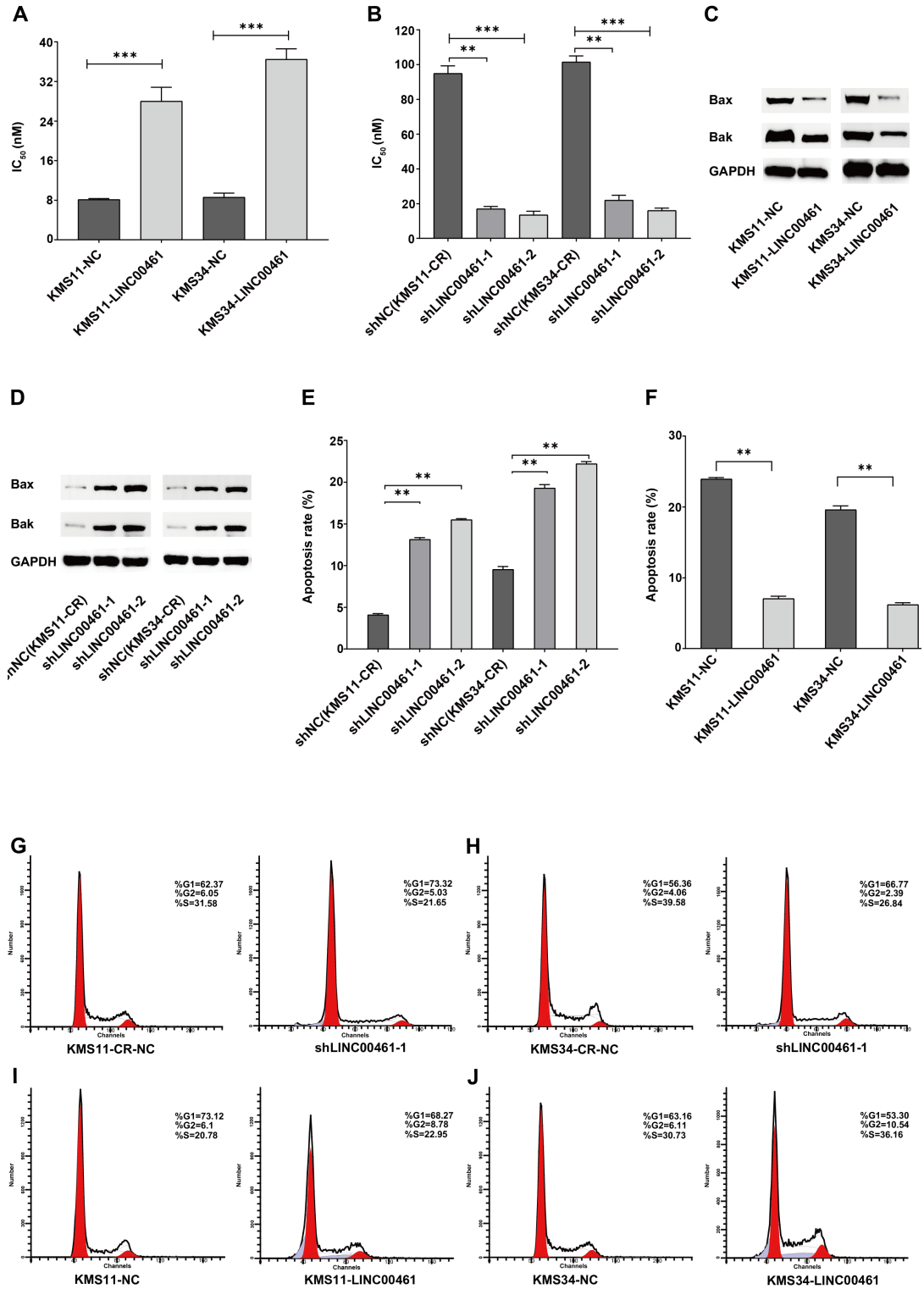

Fig. 2. LINC00461 induces carfilzomib resistance.

(A) CCK-8 experiments showed enhanced carfilzomib resistance when LINC00461 was overexpressed in KMS34 or KMS11 cells. (B) The IC_{50} of LINC00461 in KMS34-CR or KMS11-CR cells in the LINC00461-knockdown group. (C) WB experiments showing the levels of apoptotic proteins in carfilzomib-resistant cells. (D) WB experiments showing the levels of apoptotic proteins in carfilzomib-sensitive cells. (E) Apoptosis ratio of the carfilzomib-resistant cells in the shLINC00461 and control groups. (F) Apoptosis ratio of the carfilzomib-sensitive cells in the LINC00461-overexpressing and control groups. (G) Cell cycle distribution of the shLINC00461 and control groups of carfilzomib-resistant KMS11-CR cells. (H) Cell cycle distribution of the shLINC00461 and control groups of carfilzomib-resistant KMS34-CR cells. (I) Cell cycle distribution of KMS11 cells in the LINC00461-overexpressing and control groups. (J) Cell cycle distribution of KMS34 cells in the LINC00461-overexpressing and control groups. $^{**}P < 0.01$, $^{***}P < 0.001$.

LINC00461-knockdown cells (Fig. 2D). As shown in Fig. 2E, LINC00461 depletion accelerated apoptosis in carfilzomib-resistant cells, while LINC00461 overexpression inhibited apoptosis in parental cells (Fig. 2F). Consistently, silencing LINC00461 inhibited cell cycle progression from the G_0/G_1 to S phase in carfilzomib-resistant cells (Fig. 2G, H), leading to stagnation of cell growth. In contrast, overexpression of LINC00461 promoted cell cycle progression in KMS11 cells (Fig. 2I). The G_0/G_1 phase fraction of KMS34 cells with upregulated LINC00461 expression was lower than that of control cells, but the opposite was observed for the G_2/M phase fraction (Fig. 2J). Collectively, these findings suggest that LINC00461 enhances chemoresistance by modulating cell proliferation, reducing cell apoptosis and promoting cell cycle progression.

LINC00461 regulates carfilzomib sensitivity in vivo

To further confirm the effects of LINC00461 in vivo, shLINC00461 cells were injected subcutaneously into nude mice. We found that among the carfilzomib-treated mice in the LINC00461 knockdown group, the tumour volume and weight were noticeably lower than those in the group in which LINC00461 was not knocked down (Fig. 3A-C). Because miR-539-3p could interact with LINC00461 in subsequent experiments, a miR-539-3p inhibitor was added for in vivo experiments. As shown in Fig. 3D-F, the shLINC00461+miR-539-3p inhibitor group had increased tumour growth compared with that of the control group after treatment with carfilzomib. According to the histochemical analysis of the levels of cell cycle- and cell apoptosis-associated proteins, as shown in Fig. 3G-I, the levels of the Bax and Caspase3 proteins were markedly increased in mice in the LINC00461 knockdown group treated with carfilzomib, in contrast to the low levels of Ki67 in the PBS treatment group. As shown in Fig. 3J, RAB5A was markedly downregulated in the shLINC00461 group, while silencing miR-539-3p weakened the inhibitory effect of decreased LINC00461 on RAB5A, showing that a decrease in LINC00461 promoted the expression of miR-539-3p and decreased the expression of RAB5A. All the experimental results indicated the strong impact of LINC00461 on tumour cell growth and metabolism.

miR-539-3p was a direct target of LINC00461

An increasing number of studies have shown that lncRNAs can act as ceRNAs to sponge miRNAs by combining with miRNA response elements. To test this hypothesis, the TargetScan (https://www.targetscan.org/) and miRanda (https://www.mirbase.org/) prediction algorithms were used to predict the miRNAs that interact with LINC00461. The top five upregulated miRNAs were miR-539-3p, miR-323b-3p, miR-15a, miR-485-3p, and miR-324-3p. As shown in Fig. 4A, we incubated KMS11 cells with biotin-labelled LINC00461 and extracted RNA for qRT–PCR assay evaluation. The results showed that compared to that in control group, the increase was only greater in the miR-539-3p group, while there were no obvious changes in the other miRNA groups. Furthermore, miR-539-3p was identified as a target of LINC00461 in the RIP assay (Fig. 4B). In addition, miR-539-3p was upregulated only when LINC00461 was silenced in carfilzomib-resistant cells (Fig. 4C,D). Next, a mutated sequence in LINC00461 was identified and the miR-539-3p binding site was examined (Fig. 4E). To verify the interaction between LINC00461 and miR-539-3p, we performed a luciferase reporter assay, and the results showed that miR-539-3p significantly reduced the luciferase activity of the WT reporter gene but not that of the mutant gene (Fig. 4F, G). In carfilzomib-resistant cell lines, cell viability was elevated faster by miR-539-3p knockdown than in the control (Fig. 4H). Thus, LINC00461 may regulate the expression of miR-539-3p to restrict cell activity by acting as a ceRNA.

LINC00461 regulates RAB5A by sponging miR-539-53p

By exploring bioinformatics prediction tools (TargetScan, miRcode, and miRDB), we found that there were three mRNAs that may interact with miR-539-3p (Fig. 5A). As shown in Fig. 5B, RAB5A was identified as a putative miR-539-3p target among these related mRNAs in MM cells. Furthermore, miR-539-3p mimics or inhibitors modulated the effect of LINC00461 on RAB5A levels (Fig. 5C, D). Further analysis revealed that miR-539-3p mimics were able to bind to the WT version of the RAB5A 3'UTR, and this effect was reversed by the addition of LINC00461 (Fig. 5E). As shown in Fig. 5F, downregulation of miR-539-3p or overexpression of RAB5A rescued the functional depletion caused by LINC00461 knockdown in carfilzomib-resistant cells, while the enhanced IC_{50} value of LINC00461 was eliminated by overexpression of miR-539-3p or downregulation of RAB5A (Fig. 5G). Finally, the colocalization of LINC00461 and RAB5A was verified by immunofluorescence experiments (Fig. 5H). Thus, miR-539-3p and RAB5A could cooperate with LINC00461 to mediate carfilzomib resistance in MM.

c-Jun can activate LINC00461

To study the transcription factors responsible for the upregulation of LINC00461, we used the biotin-labelled LINC00461 promoter to pull down the nucleoproteins of KMS11-CR cells and analysed them by mass spectrometry. Next, 8 transcription factors (PAX5, TFEB, Foxp3, HSF1, CTCF, GATA-1, c-myc, and c-Jun) whose expression was significantly altered (fold enrichment) were screened in carfilzomib-resistant cells (Fig. 6A). As shown in Fig. 6B, C, the mRNA and protein levels of c-Jun increased in carfilzomib-resistant cells. Additionally, the results shown in Fig. 6D,E revealed that the expression of LINC00461 was attenuated or activated by the abnormal expression of c-Jun. All of these findings suggested that c-Jun could activate the transcription of LINC00461.

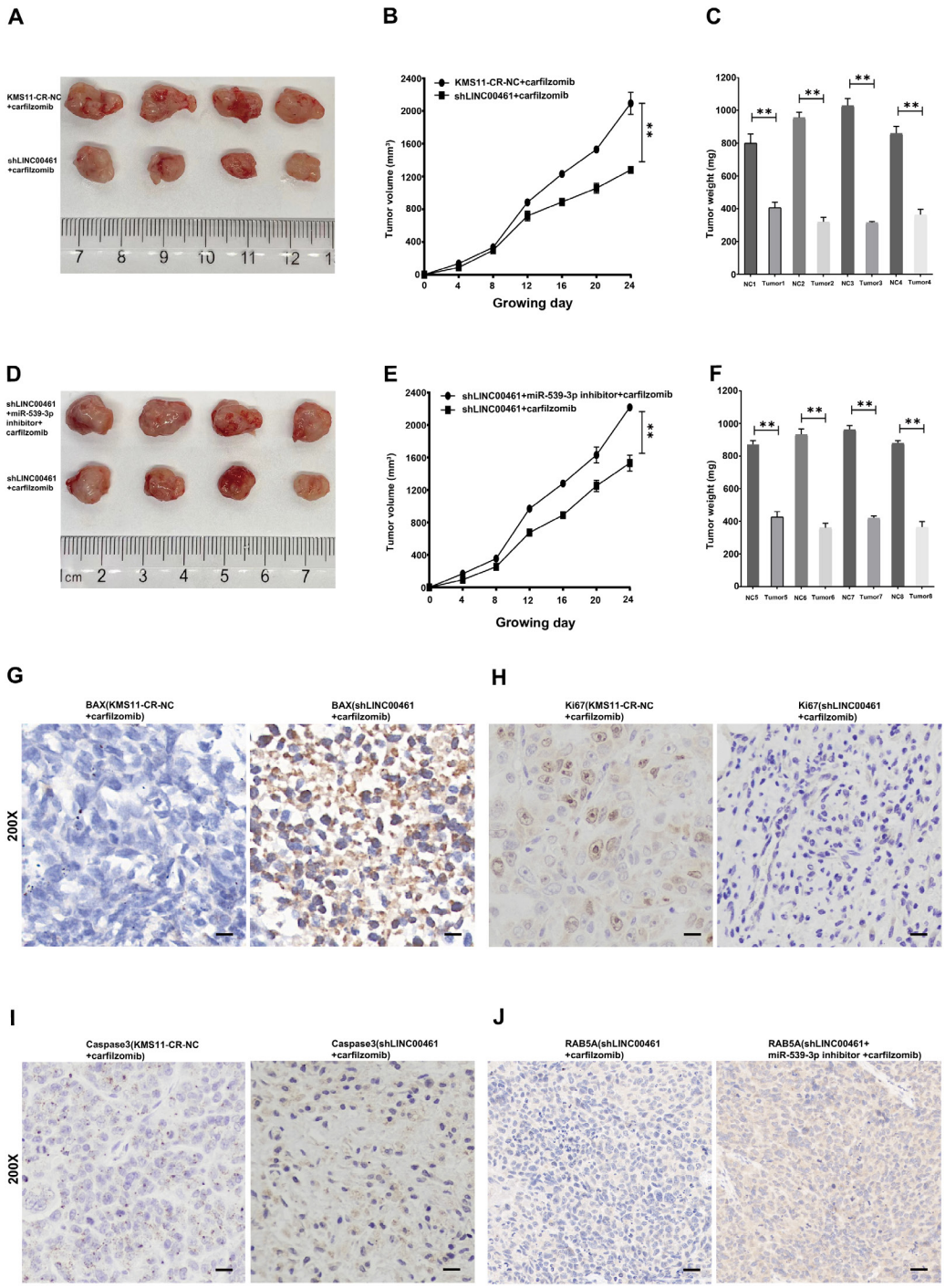

Fig. 3. LINC00461 regulates carfilzomib sensitivity.

(A) Images of subcutaneous tumours in nude mice in the shLINC00461+carfilzomib and KMS11-CR-NC+carfilzomib groups. (B) Subcutaneous tumour volume of nude mice in the shLINC00461+carfilzomib and KMS11-CR-NC+carfilzomib groups. (C) Subcutaneous tumour weight of nude mice in the shLINC00461+carfilzomib and KMS11-CR-NC+carfilzomib groups. (D) Subcutaneous tumour images of nude mice in the shLINC00461+carfilzomib and shLINC00461+miR-539-3p inhibitor+carfilzomib groups. (E) Subcutaneous tumour volume of nude mice in the shLINC00461+carfilzomib and shLINC00461+miR-539-3p inhibitor+carfilzomib groups. (F) Subcutaneous tumour weight of nude mice in the shLINC00461+carfilzomib and shLINC00461+miR-539-3p inhibitor+carfilzomib groups. (G) Histochemical images of BAX in the shLINC00461+carfilzomib and KMS11-CR-NC+carfilzomib groups. Scale bar, 200 μm. (H) Histochemical images of Ki67 expression in the shLINC00461+carfilzomib and KMS11-CR-NC+carfilzomib groups. Scale bar, 200 μm. (I) Histochemical images of Caspase3 expression in the shLINC00461+carfilzomib and KMS11-CR-NC+carfilzomib group. Scale bar, 200 μm. (J) Histochemical images of RAB5A in the shLINC00461+carfilzomib and shLINC00461+miR-539-3p inhibitor+carfilzomib groups. Scale bar, 200 μm. $^{**}P < 0.01$.

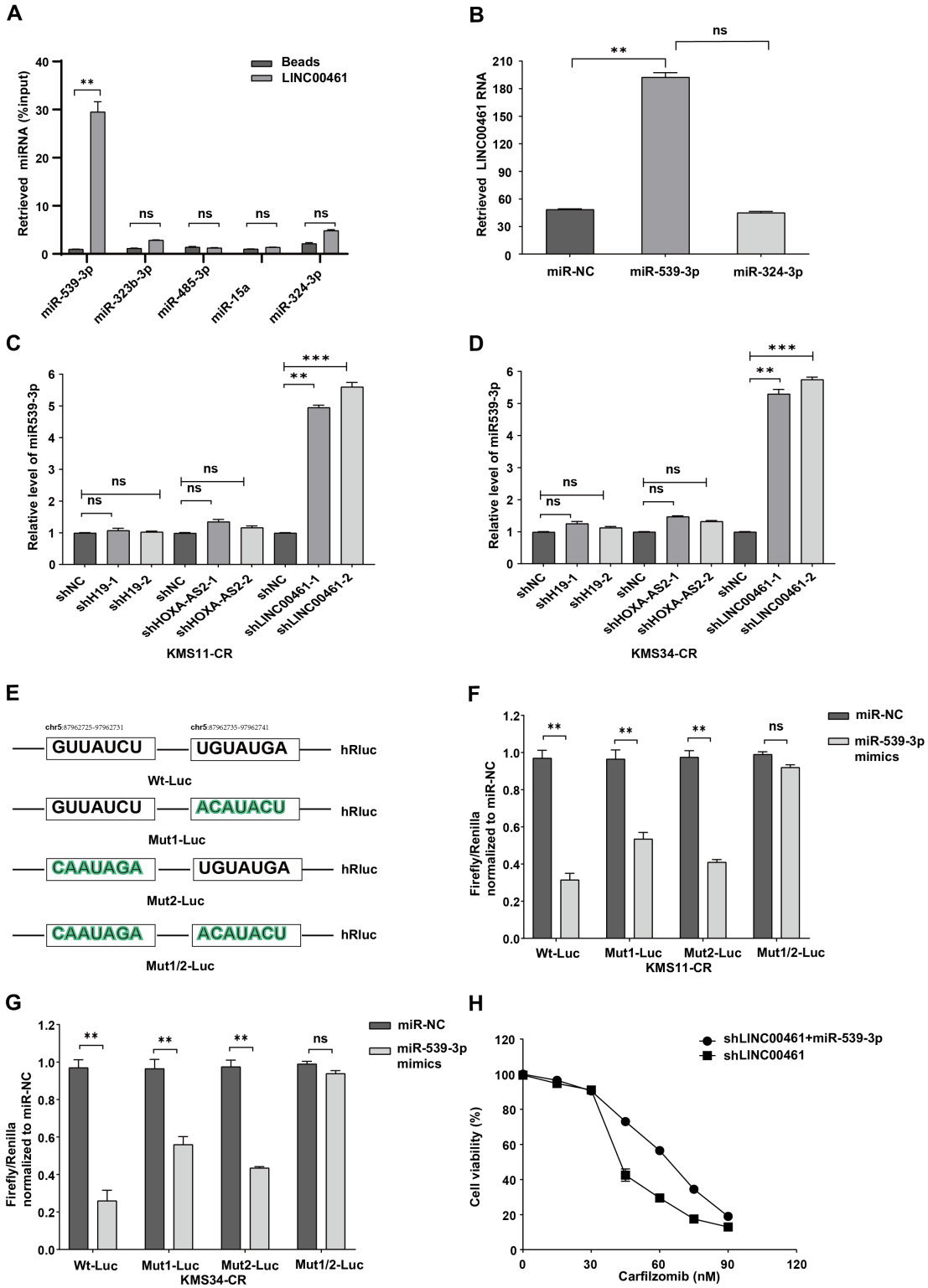

Fig. 4. miR-539-3p as a target of LINC00461.

(A) qRT-PCR was used to evaluate the expression of labeled LINC00461 in the miR-539-3p, miR-323b-3p, miR-485-3p, miR-518a-3p and miR-518c-5p groups. (B) RIP experiments showed increased expression of LINC00461 labelled with miR-539-3p. (C) Detection of the effect of three lncRNAs on the miR-539-3p expression level in KMS11-CR cells. (D) Detection of the effect of three lncRNAs on the miR-539-3p expression level in KMS34-CR cells. (E) Wild-type and mutant sequences of LINC00461 and miR-539-3p. (F) Luciferase reporter assays were conducted with LINC00461 and miR-539-3p in KMS11-CR cells. (G) Luciferase reporter assays were conducted with KMS34-CR cells transfacted with miR-539-3p mimics and the control group. (H) CCK-8 experiments showing enhanced carfilzomib resistance in the shLINC00461+carfilzomib group. ns, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

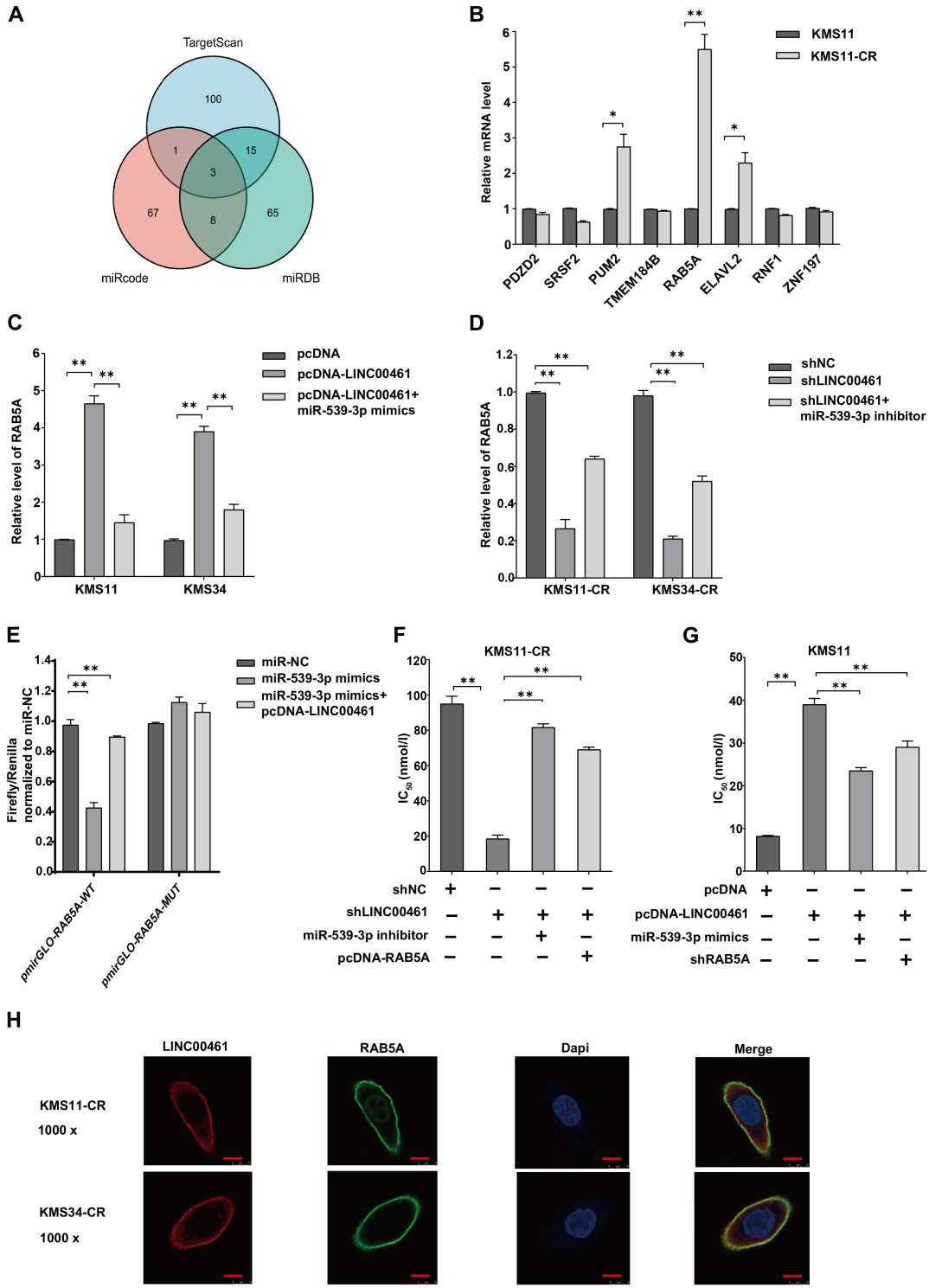

Fig. 5. LINC00461 regulates RAB5A by miR-539-53p.

(A) Interacting mRNAs identified by three online analysis tools. (B) mRNA levels of genes that interact with LINC00461 in carfilzomib-resistant cells and carfilzomib-sensitive cells. (C) mRNA level of RAB5A in the pcDNA-LINC00461, pcDNA-LINC00461+miR-539-3p mimic and control groups. (D) mRNA level of RAB5A in the shLINC00461, shLINC00461+miR-539-3p inhibitor and control groups. (E) A luciferase reporter assay was performed to confirm the associations among miR-539-3p, LINC00461 and RAB5A. (F) A CCK-8 assay was conducted to determine the change in the sensitivity of KMS11-CR cells to carfilzomib among the shLINC00461, miR-539-3p inhibitor, pcDNA-RAB5A and control groups. (G) A CCK-8 assay was conducted to determine the change in the sensitivity of KMS11 cells to carfilzomib among the pcDNA-LINC00461, miR-539-3p mimic, shRAB5A and control groups. (H) Colocalization images of LINC00461 and RAB5A in carfilzomib-resistant cells. Scale bar, 10 μm. *$P < 0.05$, **$P < 0.01$.

A

LINC00461 pull-down	Fold enrichment	RNA binding domians
c-Jun	22	4
PAX5	18	2
TFEB	15	1
Foxp3	12	0
HSF1	11	1
CTCF	9	2
GATA-1	8	4
c-myc	6	4

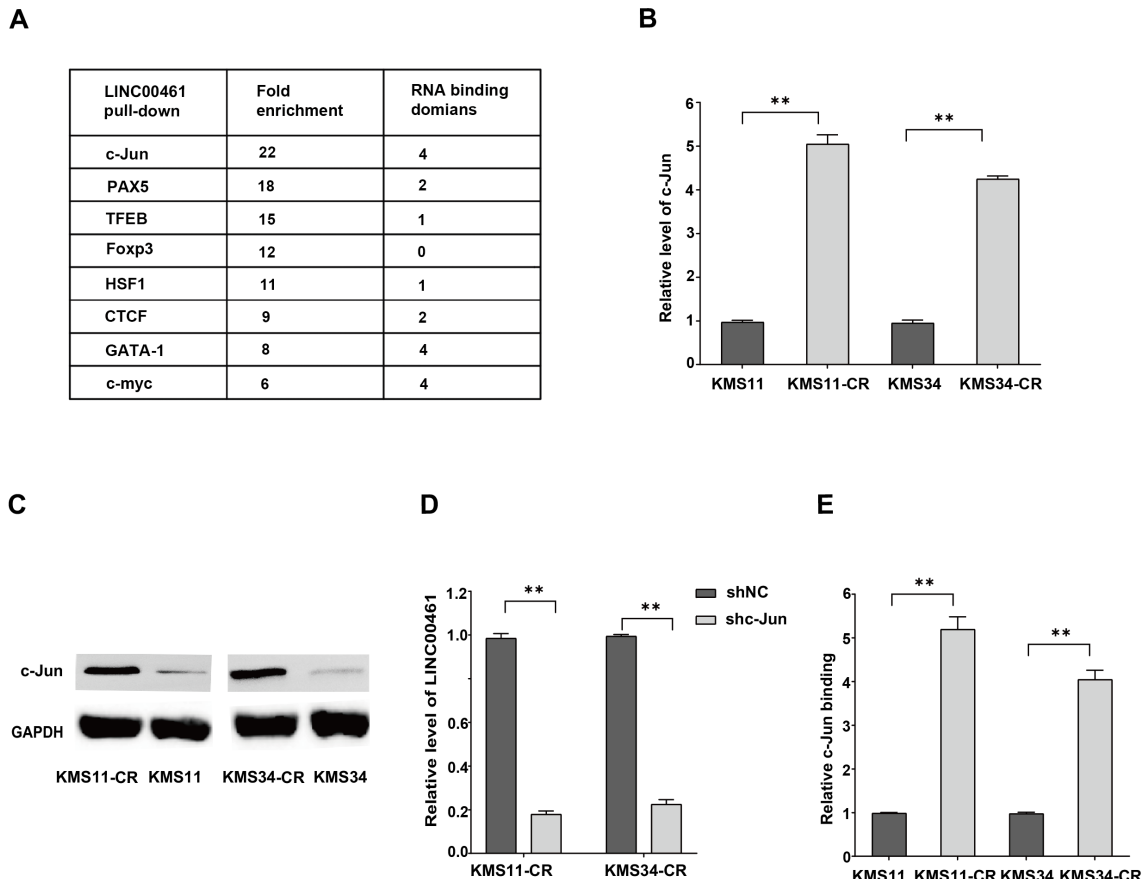

Fig. 6. c-Jun activates LINC00461.
(A) The eluted proteins labelled with LINC00461 were analysed by mass spectrometry. (B) qRT-PCR was used to compare the expression of c-Jun between carfilzomib-resistant cells and parental cells. (C) Western blotting was used to compare the expression of c-Jun between carfilzomib-resistant cells and parental cells. (D) Analysis of LINC00461 expression between shc-Jun and control group using qRT-PCR. (E) ChIP experiments were used to determine the affinity of the LINC00461 promoter for c-Jun. $^{**}P < 0.01$.

LINC00461 promotes exosome secretion in MM cells

Given that RAB5A is a pivotal regulator of exosome secretion, we analysed whether LINC00461 could affect resistance by modulating tumour-derived exosome secretion. Exosomes are membrane vesicles involved in intercellular communication and are multipurpose mediators of multiple diseases. To further analyse the role of exosomes in MM, the morphologies of exosomes isolated from carfilzomib-resistant cells were examined and are shown in Fig. 7A. The exosomes produced from KMS34-CR cells had a mean diameter of 100 nm (Fig. 7B). Next, we confirmed the presence of the exosomal protein markers CD63, HSP70, and CD9 (Fig. 7C). As shown in Fig. 7D, E, the exosomes were labelled with green fluorescent dye and cocultured with MM cells for 24 h, and the results suggested that carfilzomib-sensitive cells could more effectively ingest the tumour-derived exosomes than carfilzomib-resistant cells. As expected, we concluded that the overexpression of LINC00461 could progressively increase exosome secretion.

Discussion

MM is an aggressive cancer that accounts for more than 10% of all haematologic cancers. Carfilzomib resistance, whether primary or acquired, is the bottleneck of MM therapy. LncRNAs are quickly emerging as targets of interest as master regulators of gene expresssion, but very little is currently known about MM chemoresistance. Accumulating evidence suggests that lncRNAs represent potential targets for overcoming drug resistance in MM (Malek et al. 2016). In our study, LINC00461 was significantly more highly expressed in carfilzomib-resistant cells than in control cells.

LncRNAs have gained considerable attention due to their participation in cancer progression, tumour metastasis, and drug resistance (Huarte 2015). Wu et al. (2022) reported that LINC00461 was upregulated in MM cells. Similarly, we found increased LINC00461 expression in carfilzomib-resistant MM cells. In a similar study, Deng and colleagues (2019) characterized the promoting role of LINC00461 in promoting MM. Furthermore, Deng et al. (2023) reported that LINC00461 deficiency could restore

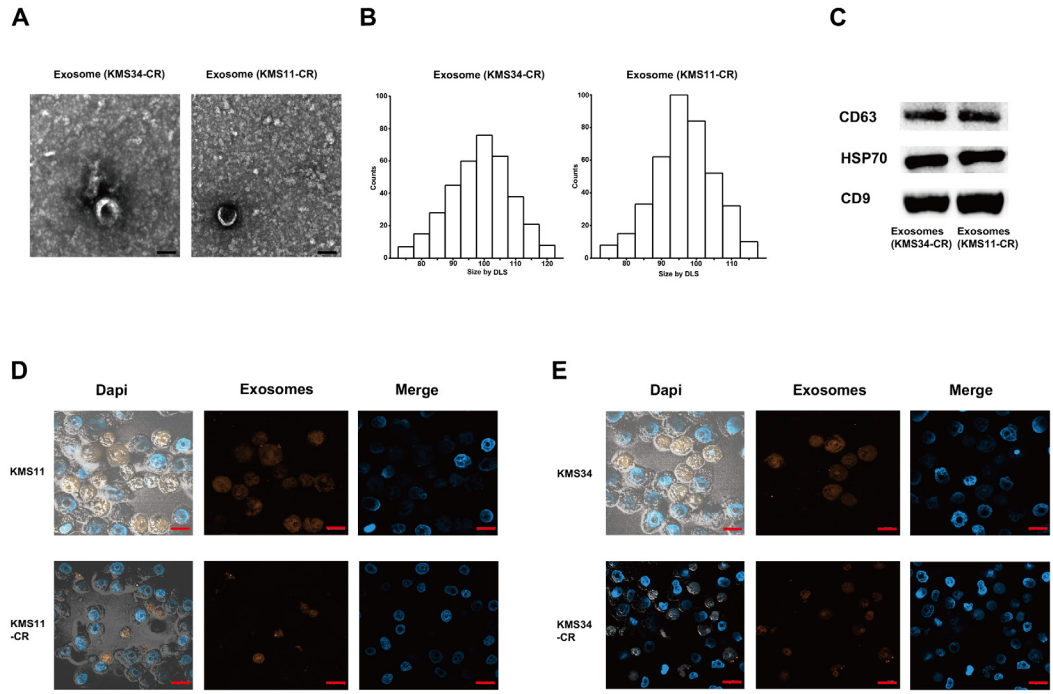

Fig. 7. LINC00461 promotes exosome secretion.
(A) Electron microscopy images of exosomes from KMS11 and KMS11-CR cells. Scale bar, 50 nm. (B) The exosomes size distribution is displayed. (C) Western blot analysis of the protein markers CD63, HSP70, and CD9 in tumour-derived exosomes. (D) Exosome uptake was compared between KMS11 and KMS11-CR cells. Scale bar, 10 μm. (E) Exosome uptake was compared between KMS34 and KMS34-CR cells. Scale bar, 10 μm.

the anti-MM efficacy of ixazomib, and silencing LINC00461 inhibited the progression of carfilzomib resistance in this study. Using flow cytometry and immunohistochemistry assays, we confirmed that LINC00461 affects drug resistance by interfering with cell proliferation and apoptosis. In accordance with the above reports, Hou et al. (2020) reported that decreased LINC00461 induces the expression of Bax, which is associated with an apoptotic phenotype in radiosensitive lung adenocarcinoma. Other studies have shown that after LINC00461 is downregulated, Ki67-induced proliferation (Sun et al. 2022) and Caspase3-dependent apoptosis increase. Interestingly, downregulated LINC00461 can hijack G_0/G_1 conversion (Yang et al. 2017), and this paper also reported the same inhibitory effect. In summary, we have provided detailed information on the role of LINC00461 in carfilzomib resistance.

Future efforts should focus on investigating the functional role of LINC00461 in chemotherapy. In general, lncRNAs can act as competitive endogenous RNAs to upregulate target mRNAs by sponging miRNAs. Concurrent with the observations of this study, LINC00461/miR-4478/E2F1 have been shown to promote the proliferation and metastasis of non-small cell lung cancer cells (Meng et al. 2020), and LINC00461/miR-15a/BCL-2 is present in MM. Moreover, using RIP experiments we confirmed that LINC00461 bound to miR-539-3p, thus promoting the process of MM chemotherapy. One clinical study revealed that miR-539-3p is an endogenous short noncoding RNA that is widely involved in vital cellular, physiological, and pathological processes in tumours. miR-539-3p promotes the progression of epithelial ovarian cancer (Gong and Fan 2019) but inhibits the proliferation and invasion of gastric cancer (Zhou et al. 2019) and colon cancer cells (Wang et al. 2020). Moreover, miR-539 inhibits cisplatin resistance by targeting doublecortin-like kinase 1 in non-small cell lung cancer. Forced miR-539 expression resensitises hepatocellular carcinoma cells to arsenic trioxide resistance (Zhu et al. 2016). However, there have been no reports of the function of miR-539-3p in MM chemotherapy. The findings of this paper indicate that miR-539-3p not only accelerates tighter binding with LINC00461 but also induces a carfilzomib response. Thus, miR-539-3p is a good candidate biomarker and driver of drug resistance in MM.

Next, bioinformatics tools and subsequent experiments demonstrated that RAB5A could bind to miR-539-3p to interfere with the growth of MM cells. A previous study revealed that RAB5A predicts the metastasis and prognosis of colorectal cancer (Yu et al. 2015). As reported by Xu, knocking down RAB5A reverses cisplatin resistance in gastric cancer (Xu et al. 2018). Additionally, some authors have reported that targeting RAB5A can serve as a strategy to overcome multidrug resistance in cancer cells (Yousaf and Ali 2020). All the above reports provide evidence that

RAB5A is an essential oncogenic factor. Furthermore, rescue technology revealed that the inhibitory effects of LINC00461 were attenuated by upregulation of miR-539-3p or knockdown of RAB5A in carfilzomib-resistant cells. Therefore, a LINC00461/miR-539-3p/RAB5A regulatory network was established.

Several studies have reported that exosome biogenesis-related factors, such as RAB5a, RAB11a, and RAB35, are enriched in exosomes (Lischnig et al. 2022). In this sense, silencing RAB5A decreases exosome secretion from hepatocellular carcinoma cells (Gorji-Bahri et al. 2021). Based on the above findings, our study was extended to determine how LINC00461 leads to exosome release. In prior reports, exosomes were described as 4-150 nm vesicles that have the ability to deliver drugs and inhibit tumour growth (Kamerkar et al. 2017). The present findings revealed that tumour-derived exosomes confer resistance to sensitive cells. Some scholars have reported that exosomal cargo containing miRNAs, mRNAs and drugs can be delivered to recipient cells to sustain chemoresistance (Mashouri et al. 2019). Li et al. (2022) also observed that tumour-derived exosomes could transmit sensitive cancer cells to a resistant phenotype via the secretion of nucleic acids and P-glycoprotein. However, some authors have shown that LINC00461 is highly expressed in exosomes derived from mesenchymal cells and enhances the proliferation of MM cells (Deng et al. 2019). Finally, challenges also exist regarding the detailed mechanism involved.

The study found that LINC00461 could affect tumor cell proliferation, invasion, metastasis, and apoptosis by ceRNA network in various tumors (Zhang et al. 2022). In MM, LINC00461 is a potential molecular biomarker in this article. Moreover, this study revealed a new regulatory mechanism by which the LINC00461-miR-539-3p-RAB5A pathway modulates carfilzomib resistance. Cumulatively, a valid therapeutic method for overcoming carfilzomib resistance has not been established, and new strategies targeting LINC00461 need to be developed to enhance the clinical benefit of carfilzomib therapy in MM patients. To gain new insights into other lncRNAs invovled in carfilzomib resistance, additional research is needed.

Author Contributions

Lifang Cheng and Fanjuan Zhang designed the study, performed the research and analyzed the data. Fanjuan Zhang supervised the study. Fanjuan Zhang collected the data and contributed new methods and wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

References

Abdi, J., Chen, G. & Chang, H. (2013) Drug resistance in multiple myeloma: latest findings and new concepts on molecular mechanisms. *Oncotarget*, **4**, 2186-2207.

Acosta-Alvear, D., Cho, M.Y., Wild, T., Buchholz, T.J., Lerner, A.G., Simakova, O., Hahn, J., Korde, N., Landgren, O., Maric, I., Choudhary, C., Walter, P., Weissman, J.S. & Kampmann, M. (2015) Paradoxical resistance of multiple myeloma to proteasome inhibitors by decreased levels of 19S proteasomal subunits. *Elife*, **4**, e08153.

Anderson, K.C. (2004) Bortezomib therapy for myeloma. *Curr. Hematol. Rep.*, **3**, 65.

Besse, A., Stolze, S.C., Rasche, L., Weinhold, N., Morgan, G.J., Kraus, M., Bader, J., Overkleeft, H.S., Besse, L. & Driessen, C. (2018) Carfilzomib resistance due to ABCB1/MDR1 overexpression is overcome by nelfinavir and lopinavir in multiple myeloma. *Leukemia*, **32**, 391-401.

Chen, Y., He, J., Su, C., Wang, H., Chen, Y., Guo, W., Li, Y. & Ding, G. (2019) LINC00461 affects the survival of patients with renal cell carcinoma by acting as a competing endogenous RNA for microRNA-942. *Oncol. Rep.*, **42**, 1924-1934.

Deng, M., Yuan, H., Liu, S., Hu, Z. & Xiao, H. (2019) Exosome-transmitted LINC00461 promotes multiple myeloma cell proliferation and suppresses apoptosis by modulating microRNA/BCL-2 expression. *Cytotherapy*, **21**, 96-106.

Deng, M., Yuan, H., Peng, H., Liu, S., Xiao, X., Wang, Z., Zhang, G. & Xiao, H. (2023) LINC00461 Knockdown Enhances the Effect of Ixazomib in Multiple Myeloma Cells. *Curr. Cancer Drug Targets*, **23**, 643-652.

Gong, Y.B. & Fan, X.H. (2019) MiR-539-3p promotes the progression of epithelial ovarian cancer by targeting SPARCL1. *Eur. Rev. Med. Pharmacol. Sci.*, **23**, 2366-2373.

Gorji-Bahri, G., Moghimi, H.R. & Hashemi, A. (2021) RAB5A effect on metastasis of hepatocellular carcinoma cell line via altering the pro-invasive content of exosomes. *Exp. Mol. Pathol.*, **120**, 104632.

Hou, J., Wang, Y., Zhang, H., Hu, Y., Xin, X. & Li, X. (2020) Silencing of LINC00461 enhances radiosensitivity of lung adenocarcinoma cells by down-regulating HOXA10 via microRNA-195. *J. Cell Mol. Med.*, **24**, 2879-2890.

Huarte, M. (2015) The emerging role of lncRNAs in cancer. *Nat. Med.*, **21**, 1253-1261.

Kamerkar, S., LeBleu, V.S., Sugimoto, H., Yang, S., Ruivo, C.F., Melo, S.A., Lee, J.J. & Kalluri, R. (2017) Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature*, **546**, 498-503.

Li, Y., Chen, Z.K., Duan, X., Zhang, H.J., Xiao, B.L., Wang, K.M. & Chen, G. (2022) Targeted inhibition of tumor-derived exosomes as a novel therapeutic option for cancer. *Exp. Mol. Med.*, **54**, 1379-1389.

Li, Y., Sun, X., Ji, D., Kong, X., Liu, D., Zhao, Z., Yan, J. & Chen, S. (2017) Expression of Rab5a correlates with tumor progression in pancreatic carcinoma. *Virchows Arch.*, **470**, 527-536.

Lischnig, A., Bergqvist, M., Ochiya, T. & Lasser, C. (2022) Quantitative Proteomics Identifies Proteins Enriched in Large and Small Extracellular Vesicles. *Mol. Cell Proteomics*, **21**, 100273.

Ma, W., Zhao, F., Yu, X., Guan, S., Suo, H., Tao, Z., Qiu, Y., Wu, Y., Cao, Y. & Jin, F. (2020) Immune-related lncRNAs as predictors of survival in breast cancer: a prognostic signature. *J. Transl. Med.*, **18**, 442.

Malek, E., Kim, B.G. & Driscoll, J.J. (2016) Identification of Long Non-Coding RNAs Deregulated in Multiple Myeloma Cells Resistant to Proteasome Inhibitors. *Genes* (*Basel*), **7**, 84.

Mashouri, L., Yousefi, H., Aref, A.R., Ahadi, A.M., Molaei, F. & Alahari, S.K. (2019) Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. *Mol. Cancer*, **18**, 75.

Meng, Q., Liu, M. & Cheng, R. (2020) LINC00461/miR-4478/E2F1 feedback loop promotes non-small cell lung cancer cell proliferation and migration. *Biosci. Rep.*, **40**, BSR20191345.

Peng, Y., Wu, W., Shang, Z., Li, W. & Chen, S. (2020) Inhibition of lncRNA LINC00461/miR-216a/aquaporin 4 pathway

suppresses cell proliferation, migration, invasion, and chemoresistance in glioma. *Open Life Sci.*, **15**, 532-543.

Qu, W., Huang, W., Yang, F., Ju, H. & Zhu, G. (2020) Long noncoding RNA LINC00461 mediates cisplatin resistance of rectal cancer via miR-593-5p/CCND1 axis. *Biomed. Pharmacother.*, **124**, 109740.

Riz, I., Hawley, T.S. & Hawley, R.G. (2015) KLF4-SQSTM1/p62-associated prosurvival autophagy contributes to carfilzomib resistance in multiple myeloma models. *Oncotarget*, **6**, 14814-14831.

Sun, S.W., Chen, Y., Liao, H.J., Zhang, W., Xu, W.M. & He, G.Q. (2022) LINC00461 Regulates the Recurrence of Large B Cell Lymphoma through the miR-411-5p/BNIP3 Pathway. *Evid. Based Complement. Alternat. Med.*, **2022**, 9100056.

Wang, Z., Hu, T., Jin, C., Yu, J., Zhu, D. & Liu, J. (2020) The antitumor effect of miR-539-3p on colon cancer via regulating cell viability, motility, and nude mouse tumorigenicity with CDK14 inhibition. *J. Gastrointest. Oncol.*, **11**, 899-910.

Wu, Y., Zhang, Z., Wu, J., Hou, J. & Ding, G. (2022) The Exosomes Containing LINC00461 Originated from Multiple Myeloma Inhibit the Osteoblast Differentiation of Bone Mesenchymal Stem Cells via Sponging miR-324-3p. *J. Healthc. Eng.*, **2022**, 3282860.

Xu, W., Shi, Q., Qian, X., Zhou, B., Xu, J., Zhu, L., Feng, L., Jin, H. & Wang, X. (2018) Rab5a suppresses autophagy to promote drug resistance in cancer cells. *Am. J. Transl. Res.*, **10**, 1229-1236.

Yang, Y., Ren, M., Song, C., Li, D., Soomro, S.H., Xiong, Y., Zhang, H. & Fu, H. (2017) LINC00461, a long non-coding RNA, is important for the proliferation and migration of glioma cells. *Oncotarget*, **8**, 84123-84139.

Yousaf, M. & Ali, M. (2020) Modulation of ABCG2 surface expression by Rab5 and Rab21 to overcome multidrug resistance in cancer cells. *Xenobiotica*, **50**, 988-996.

Yu, M.H., Luo, Y., Qin, S.L. & Zhong, M. (2015) Increased expression of Rab5A predicts metastasis and poor prognosis in colorectal cancer patients. *Int. J. Clin. Exp. Pathol.*, **8**, 6974-6980.

Zhang, C., Wang, J., Zhang, J., Qu, H. & Tang, X. (2020) LINC00461 Overexpression Can Induce Docetaxel Resistance in Breast Cancer by Interacting with miR-411-5p. *Onco Targets Ther.*, **13**, 5551-5562.

Zhang, Q., Zhong, C., Shen, J., Chen, S., Jia, Y. & Duan, S. (2022) Emerging role of LINC00461 in cancer. *Biomed. Pharmacother.*, **152**, 113239.

Zhou, J., Su, M., Zhang, H., Wang, J. & Chen, Y. (2019) miR-539-3P inhibits proliferation and invasion of gastric cancer cells by targeting CTBP1. *Int. J. Clin. Exp. Pathol.*, **12**, 1618-1625.

Zhu, C., Zhou, R., Zhou, Q., Chang, Y. & Jiang, M. (2016) microRNA-539 suppresses tumor growth and tumorigenesis and overcomes arsenic trioxide resistance in hepatocellular carcinoma. *Life Sci.*, **166**, 34-40.

Supplementary Files

Please find supplementary file(s);
https://doi.org/10.1620/tjem.2024.J082