

β -Elemene Prevented Multidrug Resistance of Small Cell Lung Cancer Cell Line H446: Targeting the Autophagy Regulation of MiR-199a-5p is the Mechanism of Cisplatin Resistance.

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Research Article

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Abstract

Background Multidrug resistance (MDR) in small cell lung cancer (SCLC) is a clinical dilemma in chemotherapy. A natural compound, β -elemene has been found to inhibit MDR cancers. We hypothesized that β -elemene regulated cisplatin-resistant SCLC by targeting autophagy-related miRNA miR-199a-5p.

Methods We assessed the IC_{50} of multiple chemotherapeutic agents toward SCLC cells H446 and MDR SCLC cells H446/EP with or without β -elemene using CCK-8. We overexpressed (OE) and knocked down (KD) miR-199a-5p in H446 and H446/EP and determined the expression of miR-199a-5p, autophagy-related proteins, and the formation of autophagolysosomes using QPCR, western blotting, and MDC staining respectively. We validate the binding of miR-199a-5p to p62 using the Dual-Luciferase assay.

Results β -Elemene decreased drug resistance of H446 to cisplatin, etoposide, paclitaxel, epirubicin, irinotecan, and vinorelbine. Cisplatin increased LC3II/LC3I, ATG5, p62, and the formation of autophagolysosomes in H446 cells, but not in H446/EP cells, and these effects were blocked by β -elemene. The OE of miR-199a-5p reduced cisplatin-induced increase of LC3II/LC3I, ATG5, p62, and the formation of autophagolysosomes while the KD of miR-199a-5p did the opposite. The OE of miR-199a-5p reduced the luciferase signal of wild-type p62, but not that of p62 with mutations at the predicted binding site. The effect of β -elemene on cisplatin-induced autophagy was validated in OE-H446 and KD-H446/EP.

Conclusions β -Elemene prevented MDR in H446 and it prevents cisplatin resistance of H446 by targeting the autophagy regulation of miR-199a-5p.

1. Introduction

More than 80% of clinical lung cancer cases are diagnosed as non-small cell lung cancers (NSCLC) and only less than 20% as small cell lung cancer (SCLC) [1, 2], but NSCLC typically grow at a slower rate than SCLC and are difficult to be discovered until they have advanced [3]. As SCLC can be diagnosed at earlier stages, chemotherapy is the major treatment for SCLC instead of surgical treatment, resulting in more drug resistance issues [4]. Multidrug resistance (MDR) is an innate and/or acquired ability of cancer cells to survive against a wide range of chemotherapy drugs [5]. In clinical cancer chemotherapy, MDR has been one of the tough dilemmas. Cancer cells with MDR can expel the anti-cancer molecules outside the cells thereby reducing the absorption of the drug. Although research has revealed many potential mechanisms underlying MDR, the understanding of MDR is still far from complete and no effective way has been found to solve the problem of MDR ideally [6]. Autophagy has been suggested to be one of the factors that might affect MDR [7–9]. It is characterized by a self-digestion pathway that activates lysosome to degrade damaged or superfluous cell components in the cells [10, 11]. Studies have shown that autophagy prevents cells from apoptosis, hypoxia, and damage stress responses. As a complex cell behavior, autophagy involved many biological processes and might interfere with MDR pathways [12, 13].

In recent years, many naturally occurring compounds have been studied and implemented in the clinical therapy of human disease [14–18]. Accumulating evidence suggests that chemotherapy supplied by traditional medicine might achieve desirable outcomes in clinical cancer treatment, including higher efficiency and lower side-effects [19] [20]. A natural compound extracted from *Rhizoma zedoariae*, β -elemene has been used for clinical cancer treatment [21] and shown anti-drug resistance potential in cancer. β -Elemene has been shown to induce autophagy in human breast cancer cells [22], but whether it affects autophagy in lung cancer has not been studied. A previous study showed that β -elemene can target lung cancer cells and promote the polarization of macrophages from M2 to M1 to decrease inflammation [23]. In gastric cancer cells, β -elemene can suppress the metastasis of multidrug-resistant cell lines [24]. In addition, β -elemene was also found to up-regulate P53 protein expression to facilitate the release of exosomes in lung cancer cells [25]. This inferred that β -elemene might regulate the membrane system in lung cancer cells, which, we suggested might involve the lysosome membrane in autophagy. Therefore, we believe that β -elemene might regulated multidrug-resistant lung cancer cells by targeting lysosome-mediated autophagy.

A microRNA (miRNA) miR-199a-5p has been found closely related to autophagy and drug resistance [26]. This miRNA plays roles in multiple cancers, including lung cancer [27–29], laryngeal cancer [30], colorectal cancer [31], etc. A previous study has found that miR-199a-5p inhibited protective autophagy and reversed chemoresistance by regulating DRAM1 protein in leukemia cells [32, 33]. Our preliminary bioinformatics study revealed that miR-199a-5p was one of the miRNAs associated with β -elemene actions (data not shown in this paper). Therefore, we proposed that miR-199a-5p might mediate the effect of β -elemene and cisplatin on autophagy of lung cancer cells. In the present study, we explored the correlation between autophagy and MDR development in small cell lung cancer cell (SCLC) H446 cells and investigated the role of miR-199a-5p in this process. Our data supported the use of β -elemene in clinical chemotherapy for MDR and revealed the role of miR-199a-5p in autophagy regulation of MDR in SCLC.

2. Methods And Materials

2.1. Cell lines and cell culture

Small cell lung cancer cell (SCLC) line NCI-H446 [H446] (ATCC® HTB-171™) and lung microvascular endothelium HULEC-5a (ATCC® CRL-3244™) were purchased from ATCC (Washington, USA). The cell lines were cultured using the RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 37°C 5% CO₂ incubator. The multidrug resistance H446 sub-cell line H446/EP was developed from H446 with increasing concentration selection of etoposide (Sigma-Aldrich, St. Louis, MO, USA) combined with cisplatin (Sigma-Aldrich, St. Louis, MO, USA), both increased from 50 ng/ml to a final dose of 1,000 ng/ml. The H446/EP obtained were cultured in the drug-free medium for over 10 generations before the experiments [34].

2.2. Cell viability assay

Cell viability was determined using the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, St. Louis, MO, USA) assay as a previous study [35]. Briefly, cells were cultured in 96-well plates with drugs accordingly. At the endpoint of the exposure, the CCK-8 reagent (10 ml/well) was added. After 3 h of incubation at 37 °C, the absorbance at 450 nm was evaluated using a microplate reader (Bio-Rad, Model 680). The cisplatin, etoposide, paclitaxel, epirubicin, irinotecan, and vinorelbine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Cell transfection

The overexpression (OE) and knockdown (KD) of miR-199a-5p were achieved by transfection of sh-miR-199a-5p vector or miR-199a-5p expression vector into cells respectively. Briefly, the miR-199a-5p or its shRNA coding sequence was cloned into the pLV-eGFP vectors. Negative expression control vectors (OENC) and shRNA control vectors (KDNC) were also constructed with the same vector. Lipofectamine® 2000 was used to transfect the cells. The transfection was validated by observing the GFP marker in the transfected cells. The vectors were purchased and constructed by Beyotime Biotechnology (Shanghai, China).

2.4. Western blotting assay

The protein expressions were analyzed using the western blotting assay as described previously [36]. Briefly, cells were lysed in the RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) plus protease inhibitor (Pierce Protease Inhibitor Mini Tablets, Thermo Fisher Scientific, Inc., Waltham, MA, USA). SDS-polyacrylamide gel electrophoresis (PAGE, Sigma-Aldrich, St. Louis, MO, USA) electrophoresis was used to separate the proteins in samples. Then the proteins were transferred to 0.2- μ m polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The membranes were blocked with blocking buffer (Pierce™ Protein-Free Blocking Buffer, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h. The membranes were then incubated with primary antibodies at 4°C, overnight, and then secondary antibodies at room temperature for 2 h (dilution following the recommended concentration of the antibody respectively). The ECL Detection Reagent (Sigma-Aldrich, St. Louis, MO, USA) was used to visualize the target protein. The primary antibodies used in this study were as follows: LC3B (1:800, 2775S, Cell Signaling); P62/SQSTM1 (1:2500, 18420-1-AP, Protein Tech); ATG5 (1:1000, GTX113309, GeneTex), and β -Actin (1:5000, sc-1615) (Santa Cruz Biotechnology, Dallas, TX, USA). All the secondary antibodies were purchased from the Abcam (Cambridge, UK).

2.5. Autophagolysosomes observation

Monodansylcadaverine (MDC, Sigma-Aldrich, St. Louis, MO, USA) staining was used to observe autophagolysosomes as described previously [37]. Briefly, cells were cultured in a 6-well plate under testing conditions. At the endpoint of the exposure, the cells were incubated with MDC (50 μ mol/L) for 30 min at 37 °C. Then the cells were washed with precooling phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA), followed by the observation using a fluorescence microscope (GXM UltraDIGI-SBMF1, USA).

2.6. Real-time quantitative PCR.

The expression of miR-199a-5p was evaluated using real-time quantitative PCR (QPCR) as described previously [38]. Briefly, TRIzol reagent (Vazyme) was used to extract total RNA from cells following the manufacturer's instruction. The target RNAs were reverse-transcribed to cDNA using the M-MLV-Reverse Transcriptase Kit (Sigma-Aldrich, St. Louis, MO, USA). Real-time PCR analysis was performed using KiCqStart® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, St. Louis, MO, USA) with a Real-Time PCR platform (CFX96, BIO-RAD). All the PCR primers used in the study were synthesized by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The expression was normalized to RNU6-1 miRNA expression using the $\Delta\Delta$ CT method. The PCR primers sequences were as follows. miR-199a-5p F: 5'-ACACTCCAGCTGGGTGTCAGTTTGTCAAAT-3', R: 5'-TGGTGTCGTGGAGTCG-3'; RNU6-1 F: 5'-CTCGCTTCGGCAGCAC-3', R: 5'-AACGCTTCACGAATTTGCGT-3'.

2.7. Dual-Luciferase Reporter Assay.

Firefly/Renilla Dual Luciferase Assay (Sigma-Aldrich, St. Louis, MO, USA) was used to test the binding of miR-199a-5p to wild-type (WT) or mutated coding sequence of p62 as previously described [39]. Briefly, cells were plated in a 96-well plate and negative plasmids or reporter plasmids with WT or mutated p62 sequence were transfected to cells. The sequences were shown in Fig. 3G2. After 48 h transfection, cells were lysed, and the luciferase signal was measured following the protocol with the microplate reader (GXM UltraDIGI-SBMF1, USA).

2.8. Experiment repetition and statistical analysis

The CCK-assay was repeated three times with 8 biological duplications. The other experiments were repeated three times with three biological duplications. Data are presented as means \pm SD. Student's t-test or one-way ANOVA analysis was used to analyzed significance. A P-value of 0.01 or lower was considered significant.

3. Results

3.1. β -Elemene concentration screening

Concentration screening by toxicity is usually the first stop of a pharmacological study [40]. In this study, we first assessed the toxicity of β -elemene toward SCLC cells H446, drug-resistant H446/EP, and normal lung cells HULEC-5a. Results showed that β -elemene at 5 μ g/ml or lower showed no significant inhibition to cell viability of all three cell lines after 24 h of exposure. β -Elemene at 10–100 μ g/ml dose-dependently inhibited cell viability of all three cell lines with similar sensitivity of H446 and HULEC-5a, and relatively less sensitivity of H446/EP (Fig. 1A). We suggested that the MDR ability of H446/EP accounted for this difference. β -Elemene at 5 μ g/ml or lower were not toxic to cells but according to our preliminary experiments, β -Elemene at 5 μ g/ml was sufficient to affect the action of cisplatin (data not shown in this paper), therefore, we used 5 μ g/ml of β -Elemene in the subsequent study.

3.2. β -Elemene decreased the resistance of multiple chemotherapeutic agents in H446/EP

Then we did a series of cell viability assays to evaluate the IC_{50} of multiple chemotherapeutic agents in H446 and H446/EP cells with or without β -elemene. This work aimed to calculate the drug resistance index for cancer (DRIC) and investigate the effect of β -elemene on MDR. Results showed that β -elemene decreased drug resistance of cisplatin, etoposide, paclitaxal, epirubicin, irinotecan, and vinorelbine. The most remarkable DRIC decrease was from cisplatin whose DRIC decreased from 29.95 to 5.56. This decrease fold change was followed by paclitaxal, epirubicin, and irinotecan whose resistance indexes with β -elemene decreased to lower than one-third of the resistance index without β -elemene, from 5.57 to 1.48, from 9.80 to 3.06, and from 5.84 to 1.61 respectively. The resistance index of etoposide and vinorelbine with β -elemene decreased to about half of that without β -elemene, from 58.45 to 27.9 and from 2.09 to 1.05 respectively. (Table 1) These results indicated that β -elemene prevented H446 cells from the drug resistance of multiple chemotherapeutic agents. As cisplatin showed the most fold change in drug resistance index, we focused on cisplatin resistance in the subsequent study to further explored the effect of β -elemene on the drug resistance of H446 cells. In this study, cisplatin at 5 μ g/ml inhibited cell viability of H446 and H446/EP with β -elemene but not H446/EP without β -elemene, thus it is a proper model for us to study the actions of β -elemene on cisplatin effects.

Table 1
DRIC of multidrug in H446.

Drug ($\mu\text{g/ml}$)	0 $\mu\text{g/ml}$ β -Elemene			5 $\mu\text{g/ml}$ β -Elemene			
	H446	H446/EP	DRIC	H446	H446/EP	DRIC	DRIC FC
	IC ₅₀ (mean \pm SD)			IC ₅₀ (mean \pm SD)			
cisplatin	0.59 \pm 0.33	17.67 \pm 2.01 ^a	29.95	0.57 \pm 0.22	3.17 \pm 1.61 ^b	5.56	0.19
etoposide	0.38 \pm 0.19	22.21 \pm 2.12 ^a	58.45	0.45 \pm 0.23	12.56 \pm 1.67 ^b	27.9	0.47
paclitaxal	0.14 \pm 0.09	0.78 \pm 0.89 ^a	5.57	0.21 \pm 0.15	0.31 \pm 0.89 ^{bc}	1.48	0.27
epirubicin	0.61 \pm 0.22	5.98 \pm 0.89 ^a	9.80	0.69 \pm 0.32	1.11 \pm 0.59 ^{bc}	3.06	0.31
irinotecan	18.98 \pm 2.11	110.86 \pm 4.89 ^a	5.84	15.32 \pm 2.02 ^a	69.98 \pm 4.34 ^b	1.61	0.27
vinorelbine	4.45 \pm 0.98	9.32 \pm 1.99 ^a	2.09	4.95 \pm 1.22 ^a	5.19 \pm 1.15 ^b	1.05	0.50

Note: "a" compared to 0 $\mu\text{g/ml}$ β -Elemene H446, "b" compared to 0 $\mu\text{g/ml}$ β -Elemene H446/EP, "c" compared to 5 $\mu\text{g/ml}$ β -Elemene H446. SD, standard deviation; DRIC, drug resistance indices for cancer; FC, fold change.

3.3. β -Elemene promoted cisplatin-induced autophagy in H446/EP

To investigate the relation between autophagy and β -elemene actions, we determined three indicators for autophagy including the ratio of LC3II and LC3I expression, the level of ATG5, and the levels of p62. Results showed that cisplatin increased LC3II/LC3I, ATG5, and p62 in H446 cells, but not in H446/EP cells. This indicated that the drug resistance of cisplatin was resulted (at least partly) from the insensitivity of autophagy induction. In addition, β -elemene alone did not make any difference in these indicators, but when it was applied with cisplatin, it increased LC3II/LC3I, ATG5, and p62 in H446/EP cells (Fig. 1B-E). This indicated that the combination of cisplatin and β -elemene significantly increased the autophagy of multi-drug resistance sub-cell line H466/EP compared to the use of cisplatin or β -elemene alone. To observe the cell activity of autophagy in the cells, we stained the autophagolysosomes with MDC. We found that β -elemene alone failed to induce the formation of autophagolysosomes while cisplatin only induced the formation of autophagolysosomes in H446 but not in H446/EP. However, this insensitivity was eliminated by adding β -elemene to the cells: the combined use of cisplatin and β -elemene significantly induced the formation of autophagolysosomes in H466/EP (Fig. 1F). This further confirmed that β -elemene promoted cisplatin-induced autophagy in H446/EP.

3.4. MiR-199a-5p was upregulated in H446/EP

As mentioned above, miR-199a-5p was involved in MDR. To test whether it was also associated with the drug resistance of H446, we compared the expression level of it in H446 and H446/EP. Results showed that the drug resistance selection increased the level of miR-199a-5p in H446 up to 1,000 times (Fig. 2A). Such a remarkable increase in miR-199a-5p expression in H446/EP suggested that miR-199a-5p might play a potential role in the drug resistance of H446. To explore the role of miR-199a-5p, we knocked down (KD) and overexpressed (OE) miR-199a-5p in H446 and H446/EP. In H446, the knockdown slightly decreased miR-199a-5p but the overexpression increased miR-199a-5p up to 1,500 folds (Fig. 2B). In H446/EP, the knockdown decreased miR-199a-5p by more than 60% and the overexpression increased the miR-199a-5p level by 150% (Fig. 2C). If we normalized all data to the endogenous level of miR-199a-5p in H446, the overexpression increased about 1,500-fold of H446 endogenous miR-199a-5p in both H446 (from 1 fold to about 1,500 fold) and H446/EP (from about 1,000 fold to about 2,500 fold). This inferred that the exogenous miR-199a-5p expression did not interaffect its endogenous expression. This independence made it a good model for studying the effect of drugs on miR-199a-5p expression because the miR-199a-5p can be fully expressed without potential negative feedback caused by the high level of miR-199a-5p.

3.5. MiR-199a-5p regulated cisplatin-induced autophagy of H446/EP

As we suggested that the mechanism of miR-199a-5p on drug resistance of H446 was associated with the autophagy of the cells, we determined the autophagy in the miR-199a-5p knockdown and overexpressing cells with cisplatin induction using MDC staining. Results showed that the knockdown did not affect cisplatin-induced formation of autophagolysosomes in H446 but the overexpression decreased the formation of autophagolysosomes. While in H446/EP cells, where the formation of autophagolysosomes was low with cisplatin induction, the knockdown of miR-199a-5p significantly increased the formation of autophagolysosomes, but the overexpression of A549-miR-199a-5p did not affect the formation of autophagolysosomes (Fig. 2D). To further confirm the role of miR-199a-5p in cisplatin-induced autophagy, we analyzed the LC3II/LC3I ratio, ATG5, and p62 expressions. Results showed the knockdown did not affect LC3II/LC3I ratio, ATG5, and p62 in H446 but the overexpression decreased LC3II/LC3I ratio, ATG5, and p62 (Fig.E1-4). While in H446/EP cells, the knockdown of miR-199a-5p significantly increased LC3II/LC3I ratio, ATG5, and p62, but the overexpression of miR-199a-5p did not affect the LC3II/LC3I ratio, ATG5, and p62 (Fig.F1-4). These results were in line with the observation of the formation of autophagolysosomes. Therefore, the knockdown of miR-199a-5p did not affect cisplatin-induced autophagy in H446 cells, but the overexpression decreased the autophagy, while in H446/EP cells, where the cisplatin-induced autophagy was low, the knockdown of miR-199a-5p significantly increased the autophagy, but the overexpression of miR-199a-5p did not affect the autophagy. We suggested that the expression level of miR-199a-5p account for most of these results. In knockdown H446, the miR-199a-5p level was only slightly decreased thus it did not regulate cisplatin-induced autophagy significantly, while in overexpressed H446, the miR-199a-5p level was high resulting in

lower cisplatin-induced autophagy. On the other hand, in knockdown H446/EP, the miR-199a-5p level was significantly decreased thereby it increased cisplatin-induced autophagy, while for overexpression in H446/EP, the miR-199a-5p level was already high in H446/EP that a further increase of miR-199a-5p did not further inhibit cisplatin-induced autophagy.

3.6. MiR-199a-5p directly targeted the p62 gene.

As our results have shown, the expression of p62 was associated with the miR-199a-5p level. Hence, we proposed that miR-199a-5p might target the p62 gene coding sequence directly. Therefore, we invested the sequence of p62 mRNA and miR-199a-5p and predicted a potential binding site with five consecutive base pairings including 4 C-G base pairs (Fig. 2G1). To test this hypothesis, we conducted the Dual-Luciferase Reporter Assay in H446 to validate the binding of miR-199a-5p to the p62 mRNA at this predicted site. The Luciferase Reporter gene was cloned with a wild-type p62 or a p62 with mutations at the predicted site (Fig. 2G2). Results showed that the overexpression of miR-199a-5p reduced the luciferase signal of samples from cells with wild-type p62 coding sequence, but it failed to affect the luciferase signal of samples from cells with mutations at the predicted site (Fig. 2G3). This indicated that the miR-199a-5p only bond to wild-type p62 mRNA but not to mutated p62 mRNA. This experiment validated the direct binding of miR-199a-5p to p62 mRNA. We suggested that this binding leads to the subsequent degradation of the p62 mRNA, which is the mechanism for miR-199a-5p down-regulating p62 expression in H446 cells.

3.7. β -Elemene prevented the miR-199a-5p increase induced by cisplatin

As we have found that the miR-199a-5p was overexpressed in H446/EP cells compared with H446 cells, we were interested in how miR-199a-5p level altered during the development of cisplatin resistance and whether β -elemene affected this process. Therefore, we exposed the cells to cisplatin and β -elemene and observed the miR-199a-5p level at different time points in H446, H446 overexpressing miR-199a-5p, H446/EP, an H446/EP miR-199a-5p knocked down. We omitted the miR-199a-5p knocked down H446 and H446/EP overexpressing miR-199a-5p because we have demonstrated that they were not much different from their parental cells in terms of responding to cisplatin and β -elemene. Results showed that, in H446 cells, β -elemene alone showed no significant effect on miR-199a-5p expression. Although cisplatin significantly induced the increase of miR-199a-5p after 24 and 48 h, when it applied with β -elemene, miR-199a-5p did not increase miR-199a-5p (Fig. 3A). This indicated that β -elemene prevented the miR-199a-5p increase induced by cisplatin, which, we suggested, was one of the mechanisms of β -elemene preventing cisplatin resistance. Interestingly, although the H446/EP had been exposed to etoposide and cisplatin during selection, miR-199a-5p expression was further increased with cisplatin exposure (Fig. 3B). A similar effect of cisplatin was also observed in H466 overexpressing miR-199a-5p and H446/EP with miR-199a-5p knocked down (Fig. 3CD). This inferred that the exogenous miR-199a-5p expression was independent of its endogenous expression as above mentioned. We supposed that this increase might be transient and if we subsequently cultured the cells without cisplatin, it would recover to the level of 0 h which was the stable level of miR-199a-5p. In addition, our results revealed that this potential transient

increase of miR-199a-5p was not observed when β -elemene was applied alone or cisplatin combined with β -elemene in H446/EP cells (Fig. 3D). This further confirmed that β -elemene prevented the miR-199a-5p increase induced by cisplatin.

3.8. The combination of cisplatin and β -elemene decreased miR-199a-5p expression.

We have demonstrated that in H446 cells with a low level of miR-199a-5p (H446 cells and H446/EP with miR-199a-5p knockdown), β -elemene prevented the miR-199a-5p increase induced by cisplatin. Then we focused on H446 cells with a high level of miR-199a-5p (H446 cells with miR-199a-5p overexpression and H446/EP) and investigated whether the combination of cisplatin and β -elemene could decrease miR-199a-5p expression. Results showed that β -elemene alone as a control did not significantly affect miR-199a-5p expression, but the combination of cisplatin and β -elemene significantly reduced the level of miR-199a-5p in both H446 cells with miR-199a-5p overexpression and H446/EP. These results inferred that miR-199a-5p was a critical target of the β -elemene and cisplatin action.

3.9. Cisplatin and β -elemene regulated autophagy through regulating miR-199a-5p

To obtain direct evidence that β -elemene prevents cisplatin resistance of H446 by targeting the autophagy regulation of miR-199a-5p, we determined the autophagy in H446 overexpressing miR-199a-5p and H446/EP by analyzing LC3II/LC3I ratio, ATG5, and p62, and MDC staining. Results came out that in H446, the overexpression altered the response of cells to cisplatin and β -elemene. In the negative control, cisplatin alone increased LC3II/LC3I ratio, ATG5, p62, and the formation of autophagolysosomes, while with miR-199a-5p overexpressed, cisplatin increased LC3II/LC3I ratio, ATG5, p62, and the formation of autophagolysosomes only with β -elemene (Fig. 3EF). On the other hand, the autophagy indicators of H446/EP cells were sensitive to cisplatin only at the presence of β -elemene, while when miR-199a-5p was knocked down, H446/EP cells became sensitive to cisplatin without β -elemene. These results suggested an essential role of miR-199a-5p in the effect of β -elemene and cisplatin and were direct evidence supporting the conclusion that β -elemene prevents cisplatin resistance of H446 by targeting the autophagy regulation of miR-199a-5p.

4. Discussion

Small cell lung cancer is a type of highly aggressive lung cancer. As it typically causes symptoms in early-stage patients, it can be discovered at earlier stages, therefore, chemotherapy drugs are usually implemented almost throughout the entire course of treatment, causing more chance for the cells to develop MDR [41]. The doses of chemotherapy are critical for the treatment of SCLC. In this study, we conducted a series of CCK-8 assays to determine the effective doses of five commonly used chemo agents including cisplatin, etoposide, paclitaxel, epirubicin, and irinotecan with H446 and H446/EP. The CCK-8 assay was a simple-step cell viability assay with stable results [42] and has fewer steps than the MTT assay [43, 44]. Thus CCK-8 was conducive for our serious viability assay. The evaluation of IC_{50} and

DRIC revealed that β -elemene prevented H446 cells from the drug resistance of all five tested chemotherapeutic agents. These results partly accounted for some clinical SCLC cases that traditional medicine prescription of herbs containing β -elemene sensitized the prolonged chemotherapy [45]. Although clinical cancer treatment involves many drugs that might potentially make a difference such as anesthetics [46–49], we believed β -elemene might be one of the critical ones.

In the present study, we selected a commonly used chemo cisplatin to further explore the actions of β -elemene which sensitized cells to cisplatin about five folds. Many previous studies have reported that cisplatin can induce MDR in H446 cells through multiple pathways [50, 51]. In this study, we used cisplatin at 5 $\mu\text{g/ml}$ because it suppressed cell viability of H446 and H446/EP with β -elemene but not H446/EP without β -elemene, hence it can be used to compare cisplatin treatment with or without β -elemene. Our results demonstrated that the drug resistance to cisplatin was resulted (at least partly) from the insensitivity of autophagy induction. Accumulating literature reported that abnormal autophagy plays a critical role in cancer MDR development [52]. Nevertheless, to date, few researchers are studying the inhibition of autophagy in drug-resistant lung cancer cells. In the present study, we demonstrated that autophagy was involved in H446/EP, and the potential mechanism included the activation of LC3I/LC3II conversion, ATG5 expression, and p62 expression. LC3II converted from LC3I has been widely accepted to be associated with the movement of mature autophagosomes along microtubular tracks [53], while ATG5 has been one of the indicators for autophagy and it plays essential roles in the elongation and expansion of phagophore membrane. The downregulation of ATG5 could prevent the autophagosome from maturation and thereby block autophagy [54]. The p62 protein, also named SQSTM1, is involved in various signaling pathways and cellular functions including autophagy [55]. These three indicators combined with the MDC assay can be used to observed autophagy comprehensively. Our result showed that the combination of cisplatin and β -elemene can significantly increase the autophagy of multi-drug resistance sub-cell line H466/EP compared to the use of cisplatin alone.

Growing lines of evidence supported the abnormal expression of miR-199a-5p in MDR cell lines. A study showed that cisplatin induced the decrease of miR-199a-5p expression in human osteosarcoma cells MG63 [56]. Another study reported that the expression of miR-199a-5p in leukemia cells from relapsed/refractory patients was lower than that from patients with complete remission [57]. However, our results showed that H446/EP expressed a higher level of miR-199a-5p, and cisplatin induced an increase of miR-199a-5p expression in H446 after 24 h. We suggested that the reaction of miR-199a-5p expression to cisplatin was cancer-type specific. Our study was the first paper that reported an abnormally high expression of miR-199a-5p in drug resistance lung cancer cells and our study is conducive to the development of miR-199a-5p as a potential biomarker for the occurrence of drug resistance in lung cancer cells. Another striking finding of this study was that miR-199a-5p could directly bind to p62 mRNA resulting in the degradation of p62 in autophagy repressive H446/EP cells. Our data suggested that miR-199a-5p could be a pharmacological target for p62 protein and it was critical in mediating autophagy regulation of β -elemene and cisplatin. The role of the miR-199a-5p in autophagy regulation cisplatin and β -elemene was illustrated in Fig. 4. In addition, sodium homeostasis has been suggested to be involved in

autophagy, but whether the role of sodium channels in cancer [58] is associated with autophagy, β -elemene, and MDR needs further investment.

5. Conclusions

β -Elemene prevented multidrug resistance of small cell lung cancer cell line H446 and β -elemene prevents cisplatin resistance of H446 by targeting the autophagy regulation of miR-199a-5p.

6. Declarations

Ethics approval and consent to participate

This work was approved and consented by the Ethical Committee of the Second Hospital of Hebei Medical University.

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Availability of data and materials

The raw data of this study are provided from the corresponding author with reasonable request.

Authors' contributions

Tiezhi Li contributed to the design of the study and most experimental work. Helin Zhang, Zhichao Wang, Shaolin Gao, Xu Zhang, Na Wang and Haiyong Zhu contributed to the data analysis and the drafting the manuscript. Honglin Li supervised the project. All author had given final approval of the version to be published.

Competing interests

The authors claimed that there is no conflict of interest.

Consent for publication

All the author consent for this publication.

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Figures

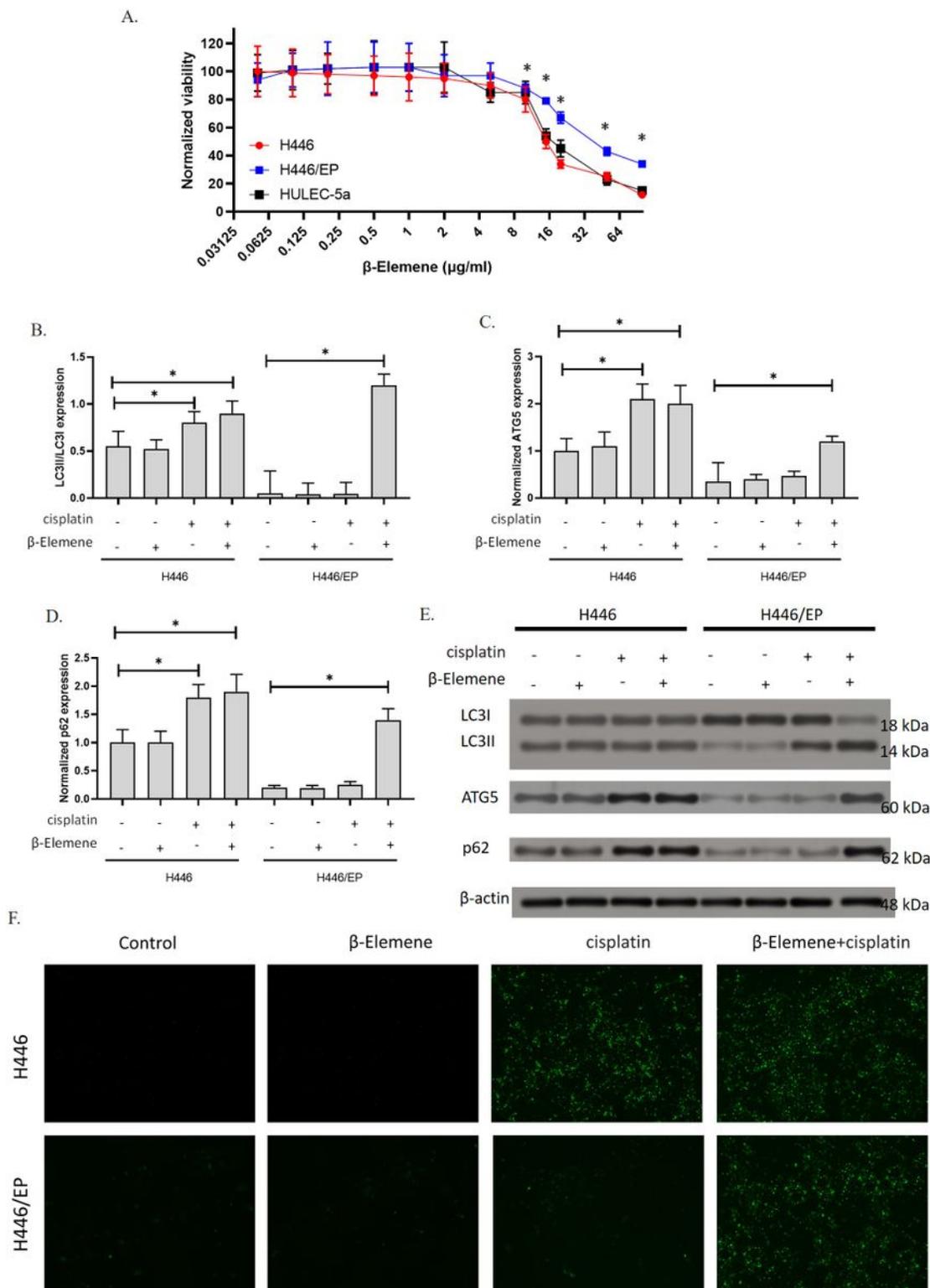


Figure 1

β -Elemene decreased DRIC of multiple chemotherapeutic agents in H446. A. Effect of β -elemene on cell viability of H446, H446/EP, and HULEC-5a. The cell viability was measured using CCK-8 24 h after exposure to the drugs. B-C. LC3II/LC3I, ATG5, and p62 expressions in H446 and H446/EP after drug exposure. The expression was measured using the western blotting assay. E. Representative images of B-

C. F. Image of autophagolysosomes in cells with drug exposure. The fluorescence images of autophagolysosomes were captured after the MDC staining of cells (magnification x200). (*p<0.01)

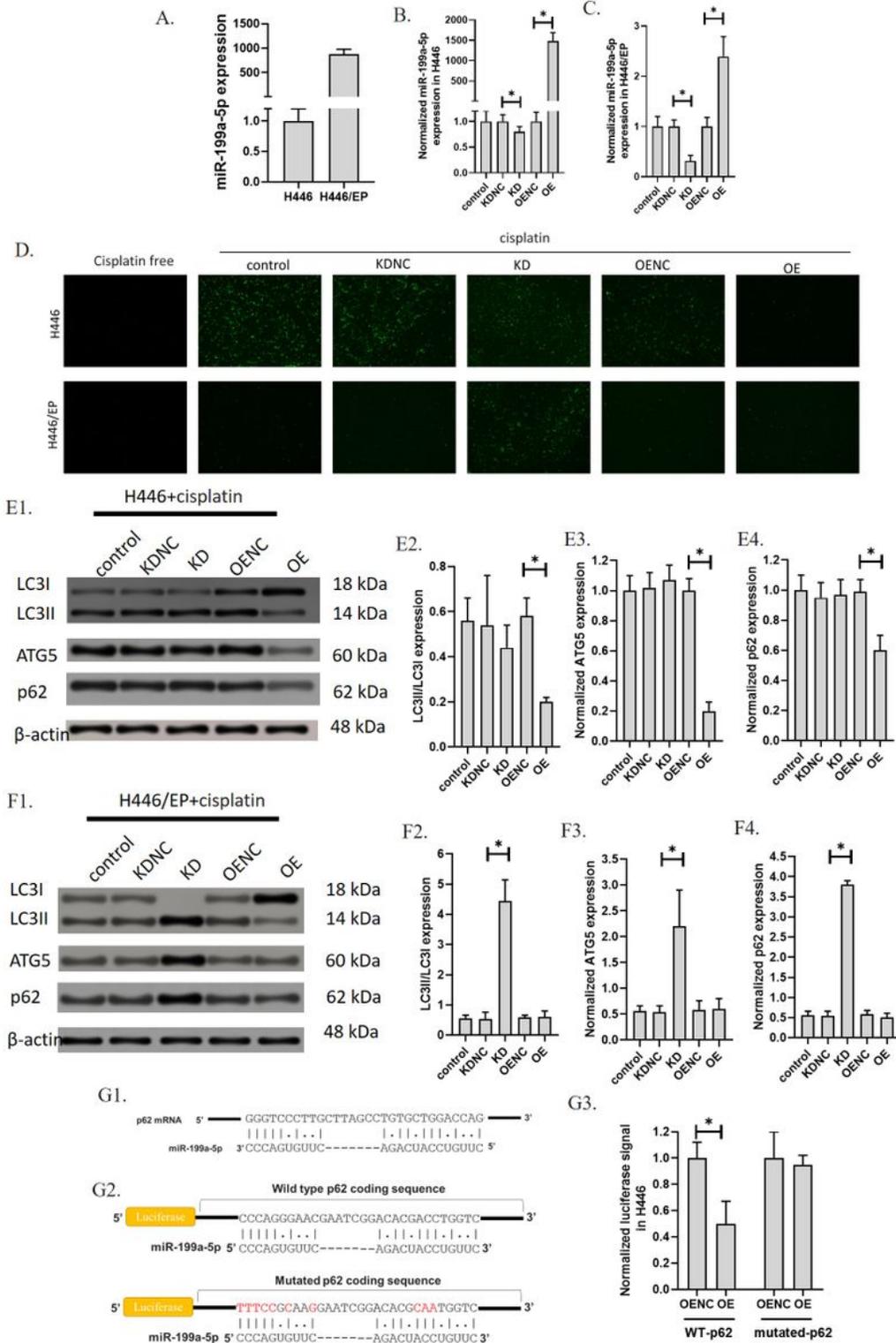


Figure 2

MiR-199a-5p regulated cisplatin-induced autophagy of H446/EP by inhibiting p62 and ATG5. A. MiR-199a-5p expression in H446 and H446/EP. MiR-199a-5p expression was determined using the QPCR assay. B. MiR-199a-5p expression in H446 with miR-199a-5p knockdown or overexpression. (OENC:

overexpression negative control; OE: MiR-199a-5p overexpression; KDNC: knockdown negative control; KD: MiR-199a-5p knockdown) C. MiR-199a-5p expression in H446/EP with miR-199a-5p knockdown or overexpression. D. Image of autophagolysosomes in miR-199a-5p knocked down or overexpressed cells with cisplatin exposure. The autophagolysosomes were observed using MDC assay (magnification x200). E1-4. LC3II/LC3I, ATG5, and p62 expressions in miR-199a-5p knocked down or overexpressed H446. The protein expression was determined using the western blotting assay. F1-4 LC3II/LC3I, ATG5, and p62 expressions in miR-199a-5p knocked down or overexpressed H446/EP. G1. The predicted binding site of miR-199a-5p to p62 mRNA. G2. Luciferase reporter gene sequence with the alignment of the miR-199a-5p gene at the predicted binding site in Dual-Luciferase Reporter Assay. G3. Effects of miR-199a-5p on luciferase signal in H446. H446 cells were co-transfected with miR-199a-5p expression vectors and wild-type (WT)-p62 or mutated-p62 vectors. The luciferase signal was determined 24 h after the transfection followed by the addition of substrate. (*p<0.01)

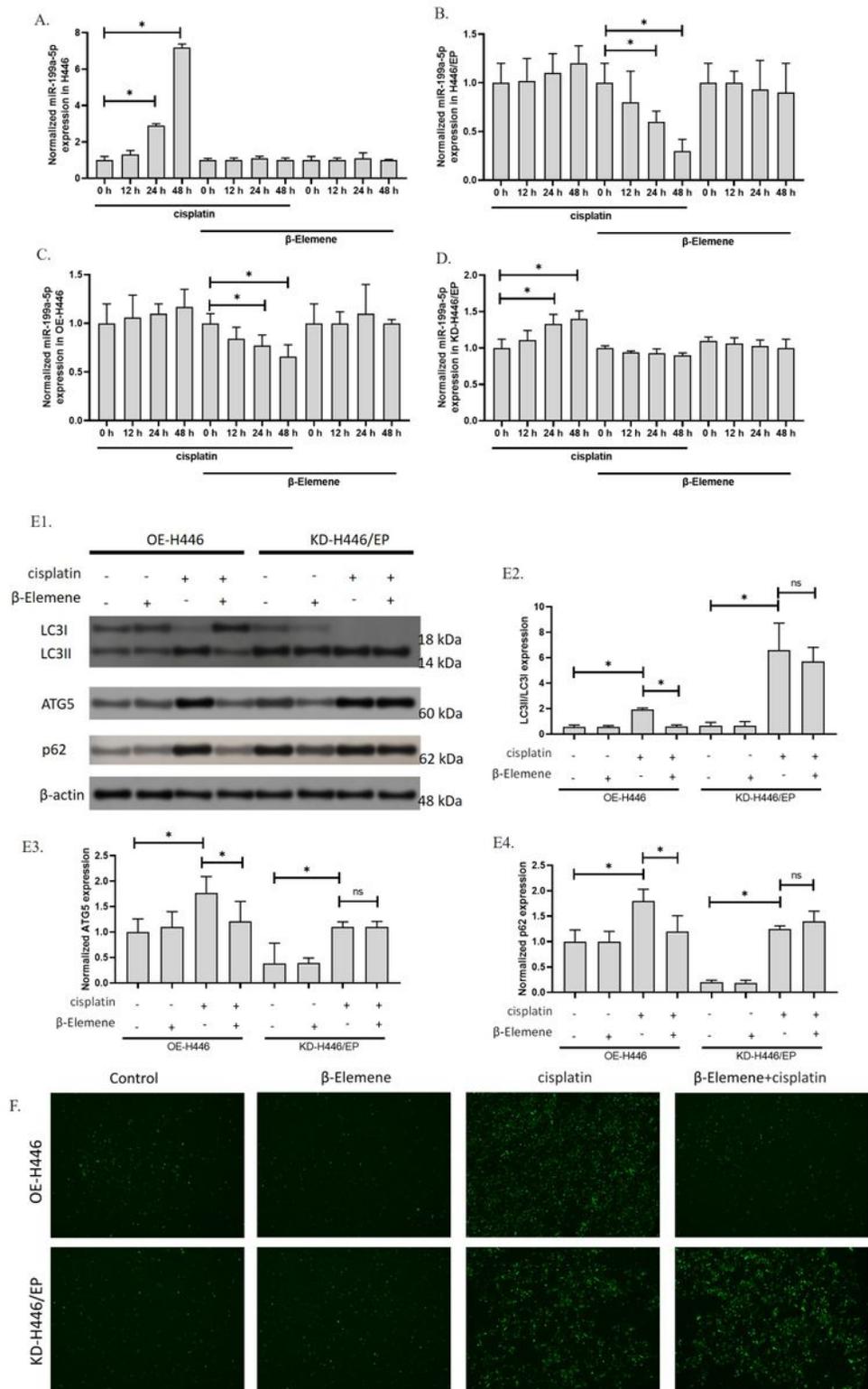


Figure 3

β -Elemene regulated the effect of cisplatin on MiR-199a-5p. A. The effect of cisplatin and β -elemene on miR-199a-5p expression in H446. MiR-199a-5p expression was determined using QPCR assay. B. The effect of cisplatin and β -elemene on miR-199a-5p expression in H446/EP. C. The effect of cisplatin and β -elemene on miR-199a-5p expression in miR-199a-5p knocked down H446/EP. D. The effect of cisplatin and β -elemene on miR-199a-5p expression in miR-199a-5p overexpressed H446. (OENC: overexpression

negative control; OE: MiR-199a-5p overexpression; KDNC: knockdown negative control; KD: MiR-199a-5p knockdown) E1-4. LC3II/LC3I, ATG5, and p62 expressions in cells. The protein expression was determined using the western blotting assay. F. Image of autophagolysosomes in miR-199a-5p knocked down H446/EP or overexpressed H446 with cisplatin and β -elemene exposure. The autophagolysosomes were observed using the MDC assay (magnification x200). (* $p < 0.01$)

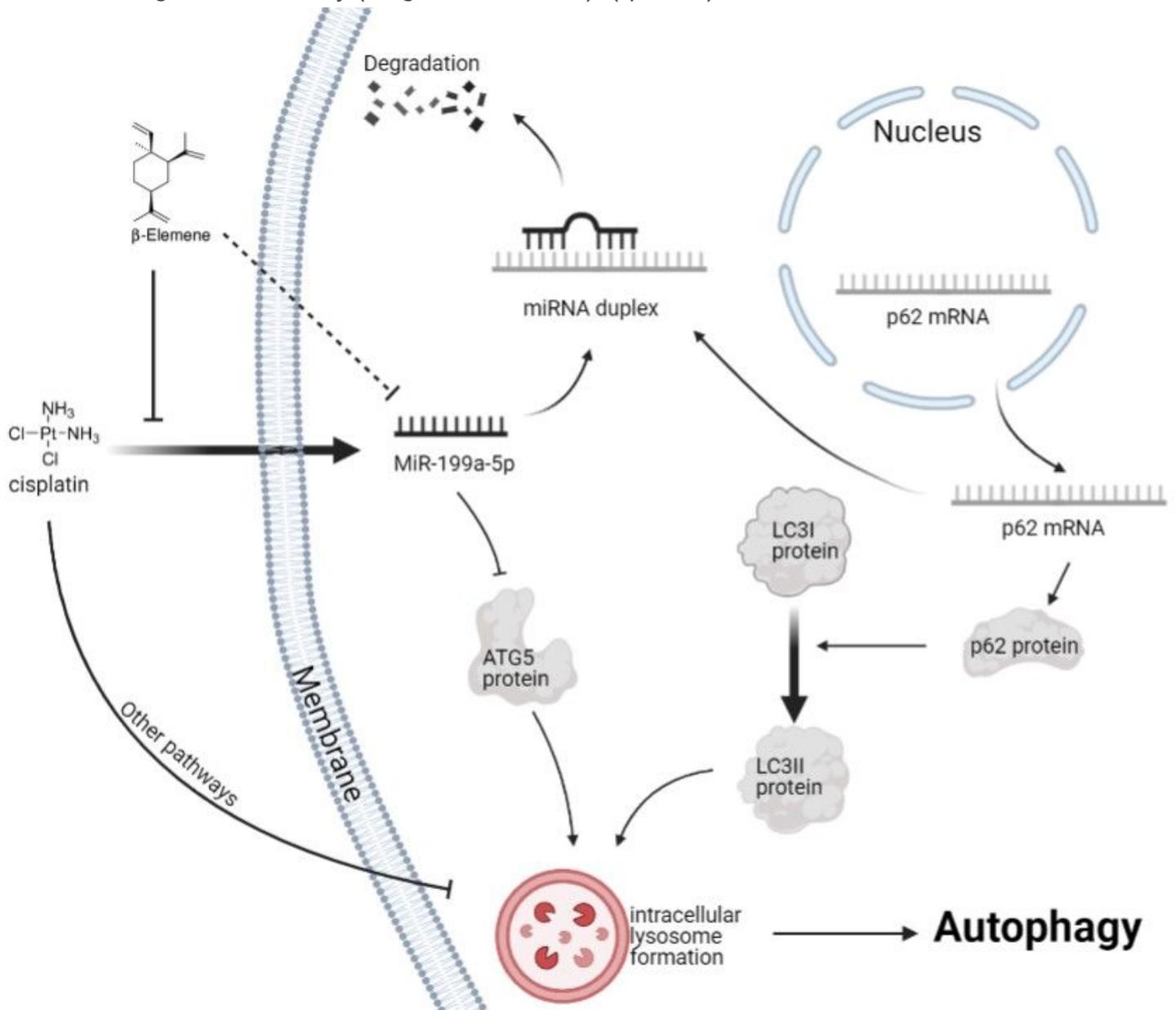


Figure 4

β -Elemene prevents cisplatin resistance of H446 by targeting the autophagy regulation of miR-199a-5p.