Chaperone-mediated autophagy affects tumor cell proliferation and cisplatin resistance in esophageal squamous cell carcinoma

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INTRODUCTION

Esophageal cancer is a malignant tumor type prevalent in developing countries, with a poor 5-year survival rate of about 30.4%.1 In China, about 90% of esophageal cancer patients are esophageal squamous cell carcinoma (ESCC) and the case number accounts for 50% of the global incidence.2 Despite continuous development of chemotherapies, including novel targeted therapies and cancer immunotherapies, the prognosis of ESCC remains poor due to diagnostic difficulty, drug resistance, and tumor recurrence. Potential new prognostic markers and therapeutic targets are urgently needed to provide better quality of clinical care for ESCC patients.

Autophagy is an important cellular process that is critical in maintaining cellular homeostasis, regulating cell differentiation, and controlling cell response to oxidative stress and nutrient starvation.3 It is usually divided into four forms: macroautophagy, selective autophagy, microautophagy, and chaperone-mediated autophagy (CMA).4

CMA is the most specific form of autophagy. There are four stages in the process of CMA. The first stage is substrate recognition and lysosomal targeting, which means that damaged or misfolded proteins with KEFRQ-like motifs...
will be recognized by a constitutive chaperone, Hsc70 and translocated to the lysosomal membrane. The second stage is substrate binding, in which the substrate is bound by the monomeric lysosomal membrane-associated protein 2a (LAMP2a) in the lysosomal membrane. After that is substrate translocation, the third stage. In this stage, monomeric LAMP2a multimerizes and forms a complex to translocate the substrate to the lysosome. The last stage is substrate degradation inside the lysosome. The substrate is hydrolyzed by proteolytic lysosomal enzymes and multimeric form LAMP2a returns to monomeric form, thus initiating a new cycle of binding and translocation. CMA provides cells with energy and important nutrition, such as amino acids and free fatty acids.\(^5\) CMA plays important roles in pathogenesis in various diseases, such as Parkinson’s disease,\(^6\) Huntington’s chorea,\(^7\) and tumor.\(^4\)–\(^11\)

LAMP2a is a membrane glycoprotein of the LAMP2 family and usually locates on the lysosomal membrane, which can bind and transport proteins on the lysosomal membrane into the lysosomal lumen.\(^12\),\(^13\) The level of LAMP2a is widely used to indicate the activity of CMA.\(^10\),\(^14\)–\(^16\)

Through immunohistochemical staining of nearly 600 tissue samples of ESCC patients, it was revealed that LAMP2a was expressed predominantly in the cytoplasm in highly differentiated or more advanced tumor node metastasis (TNM) stage tissue samples and higher expression of LAMP2a corresponded to shorter survival time.\(^17\) However, the underlying mechanisms of how CMA influences ESCC patient mortality are yet to be defined.

In this study, we modulated the activity of CMA through LAMP2a or small molecular compounds in ESCC cell lines to dissect how CMA influences the biological behavior of ESCC.

**MATERIALS AND METHODS**

**Cell lines**

The human ESCC KYSE cell lines were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, and were routinely maintained in the Roswell Park Memorial Institute 1640 (RPMI-1640) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. All cell lines were cultured at 37°C in a humidified incubator in an atmosphere containing 5% CO\(_2\). To activate CMA maximally, cells were treated with 1.5 μM AC220 (Selleckchem, s1526) and 10 μM spautin-1 (Sigma-Aldrich, SML0440) as previously described.\(^18\)

**Construction of plasmids and stable transfected cell lines**

pshRNA, psPAX2, pMD2.G, and FUB-P2A-EGFP-T2A-Puro plasmids were purchased from Addgene. Lentiviral vector harboring a shRNA sequence targeting LAMP2a was used to knockdown LAMP2a in KYSE cell lines. Lentiviral vector harboring LAMP2a cDNA sequence was used to overexpress LAMP2a in KYSE cell lines. The shRNA sequences used were as follows: shRNA forward 5'-CCGGAAGCACCATTATATCAGTGACTCAGCATGAGATAT-3' and shRNA reverse 5'-AATTTAAAAA AAGCACCATTATGCTGGATATCCTCGAGATAT-3'. AgeI (NEB) and EcoRI (NEB) restriction endonucleases were used to digest the pshRNA vector and the shRNA sequence, and they were subsequently ligated by T4 DNA ligase (NEB). For construction of LAMP2a overexpression recombinant plasmids, the cDNA of KYSE150 was used as a template to amplify LAMP2a. Homology arms were then added to the cDNA of LAMP2a using Rec-BamHI-Forward primer caggtgcagcttaggtgcgccCATCATCGTGGAGCTTCCGCGGCTTC GCT and Rec-EcoRI-Reverse primer cgtgctgccgttctcccctaaATTTGCTCATATCACCGATGACGTC. The cDNA of LAMP2a containing the homology arm was finally ligated to the linear FUB-P2A-EGFP-T2A-Puro plasmids digested by BamHI (NEB) and EcoRI (NEB) enzymes. Both the constructed knockdown and overexpression plasmids were packaged into viral particles with the help of psPAX2 and pMD2.G in Human embryonic kidney293T cells. Subsequently, ESCC cell lines were infected by viral particles and 8 μg/ml polybrene (Solarbio) was used to help viral infection.

**Real-time Polymerase chain reaction (PCR)**

Total RNA was extracted from cell lines using Total RNA Extraction Reagent (Vazyme Biotech). Total RNA (2 μg) was reverse-transcribed using a Hiscrypt II 1st Strand cDNA Synthesis kit (Vazyme Biotech). mRNA expression of LAMP2a was determined by real-time PCR using PowerUp TM SYBR TM Green Master Mix (Thermofisher Applied Biosystems). PCR amplification cycles were carried out for 2 min at 50°C and 2 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. β-actin was used as an endogenous control. Primers sequences were actin 5'-CAACAGGGAACACATCTCAGGTTTGCACGCTGAGACGGAGATGCTGACGAGATTTCGAATGTCGAGTTCTTGCAGGACTGAGAAATATCCATCTGAACACCCTGACGAGACGTAGCTGACTGACGAGATAT-3' and 5'-CCGGAAGCCACATCACATTATCAGTGACTCAGCATGAGATAT-3'. AgeI (NEB) and EcoRI (NEB) restriction endonucleases were used to digest the pshRNA vector and the shRNA sequence, and they were subsequently ligated by T4 DNA ligase (NEB). For construction of LAMP2a overexpression recombinant plasmids, the cDNA of KYSE150 was used as a template to amplify LAMP2a. Homology arms were then added to the cDNA of LAMP2a using Rec-BamHI-Forward primer caggtgcagcttaggtgcgccCATCATCGTGGAGCTTCCGCGGCTTC GCT and Rec-EcoRI-Reverse primer cgtgctgccgttctcccctaaATTTGCTCATATCACCGATGACGTC. The cDNA of LAMP2a containing the homology arm was finally ligated to the linear FUB-P2A-EGFP-T2A-Puro plasmids digested by BamHI (NEB) and EcoRI (NEB) enzymes. Both the constructed knockdown and overexpression plasmids were packaged into viral particles with the help of psPAX2 and pMD2.G in Human embryonic kidney293T cells. Subsequently, ESCC cell lines were infected by viral particles and 8 μg/ml polybrene (Solarbio) was used to help viral infection.

**Western blot analysis**

Western blot analysis was performed as follows. Briefly, proteins from total cell lysates were separated by standard SDS gel electrophoresis and transferred to PVDF membranes. The membranes were washed, blocked, and incubated with specific primary antihuman antibodies against β-actin (1:2000, TA-09; ZSGB-BIO) or LAMP2a (1:1000, ab18528; Abcam), EIF4A1 (1:500, ab31217; Abcam), RND3 (1:500, 05-723; Millipore) followed by incubation with horseradish peroxidase-conjugated secondary antibodies.

**Cell proliferation, migration, and colony formation assay**

Cell proliferation was assessed using IncuCyte ZOOM (Essen Bioscience). Briefly, cells were seeded in 96-well cell culture plates with 5 × 10\(^3\) cells/well and then cultured at
37°C in a humidified incubator with 5% CO₂. The machine monitored the cell growth continuously until the entire well bottom was totally covered by cells. Scratch-wound assay was performed to evaluate cell migration ability as previously described. Briefly, cells were cultivated in six-well plates for 24 h to form a cell monolayer, then the monolayer was wounded by a 200 μl pipette tip. The floating cells were washed away by PBS. The healing area was recorded under the microscope at 0, 24, and 48 h. The area of the scratch at each time point was calculated by ImageJ. For cell colony formation assay, 500 cells were cultured in six-well plates for 14 days to assess the number of colonies formed by tumor cells.

**Colony formation assay**

Cells were trypsinized, counted, and viability determined. Of those viable cells, 3000 were placed in a six-well plate (in triplicate). Cells were allowed to adhere and grow for about 12–14 days. To visualize colonies, cells were fixed in 4% paraformaldehyde and stained with Crystal Violet (Sigma-Aldrich). Colonies were scored manually and the percentage colony formation was calculated.

**Assessment of apoptosis**

Cellular apoptosis was evaluated by measuring the exposure of phosphatidylserine on cell membranes using Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) staining. A Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme) was used for the assessment of apoptosis. After treatment, both floating and adherent cells were collected and suspended in 100 μl of binding buffer. The cells were then incubated with 5 μl AnnexinV-FITC and 5 μl PI at room temperature for 15 min in the dark. An additional 400 μl of binding buffer was added to each tube before analysis by flow cytometry.

**Establishment of cisplatin resistant cell line**

KYSE510 cell line was cultured in 1640 medium containing 10% FBS. When the cell confluence reached about 80%, the medium was changed and 3 μmol/L cisplatin was added to it. Four hours later, the medium was discarded and changed to fresh medium without cisplatin. After the cell confluence reached about 80%, 3 μmol/L cisplatin was added to the medium again and 4 h later the medium was changed without cisplatin. This process was repeated for about 6 months to get the cisplatin resistant cell line KYSE510CDDP.

**MTS assay**

To detect the cell sensitivity to cisplatin, 8000 cells/well in the logarithmic growth phase were plated in a 96-well plate. The next day, cisplatin was added to the corresponding well. The gradient was set to 60, 30, 15, 7.5, and 3.75 μM. Forty-eight hours later, cell viability was measured with MTS at OD492nm, and IC50 was calculated by GraphPad Prism 5.

**Nude mice xenograft model**

KYSE150 and KYSE150 shLAMP2a cells were trypsinized and collected by centrifugation. Cell viability was confirmed to be above 95% based on trypan blue staining. The cells (2 × 10⁶) in 50% Matrigel (BD Biosciences) were inoculated subcutaneously (200 μl) into the right flank of 4- to 6-week-old female BALB/c nu/nu mice (purchased from Vital River Laboratory Animal Technology Co.). After inoculation, the mice were maintained under sterile conditions and the size of the tumor formed was measured using calipers twice per week. Tumor volume was calculated by the following formula: volume = (length/2) × (width²). The mice with palpable tumors were divided randomly into four groups of five mice each: (a) KYSE150 group in which 150 cells alone was received; (b) KYSE150 + cisplatine group in which 4 mg/kg cisplatin (diluted with PBS) was administered intraperitoneally once a week for 6 weeks; (c) KYSE150 shLAMP2a group in which KYSE150 shLAMP2a cells were received; (d) KYSE150 shLAMP2a + cisplatine group in which mice were treated as group 2. Animal care and experiments were conducted in accordance with the Animal Research Committee Guidelines of Zhengzhou University.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5 software. Groups of data were analyzed by Student’s t-test (two groups) or two-way ANOVA (more than two groups), p < 0.05 was considered statistically significant.

**RESULTS**

**Esophageal cancer patients with higher expression of LAMP2a exhibit poorer prognosis**

Previous research reported that higher expression of LAMP2a was associated with more advanced TNM stages and shorter survival time in ESCC patients. We validated this finding by analyzing the expression profile of LAMP2a in esophageal patients in the TCGA database by GEPIA and found that LAMP2a expressed highly in tumor tissues as compared with paired normal tissues (Figure 1a). The patients with higher expression of LAMP2a presented poorer survival time compared with those patients with lower expression of LAMP2a in this database (Figure 1b). Whether LAMP2a is engaged in the tumorigenesis or just an indicator of tumor stage still needs to be investigated.
High expression of LAMP2 indicates poor prognosis of esophageal cancer patients. (a) Analyzing the expression of LAMP2 in esophageal tumor tissues (T, red) and paired normal esophageal tissues (N, black) in the TCGA database by GEPIA, \(^*p < 0.05\). (b) Survival curves of esophageal cancer patients with high expression and low expression of LAMP2 in the TCGA database analyzed by GEPIA.

**Figure 2** Construction of LAMP2a stably knockdown or overexpressed ESCC cell lines. mRNA (a) and protein (b) expression of LAMP2a was detected in a panel of KYSE cell lines by Q-PCR and western blot. (c) Relative LAMP2a mRNA expression detected by Q-PCR in LAMP2a knockdown KYSE 150shLAMP2a and 510shLAMP2a cell lines as compared to control KYSE150 and 510 cell lines. con, control cells; KD, LAMP2a knockdown cells. \(^*p < 0.05\) compared with control cells. (d) Protein expression of LAMP2a detected by western blot in KYSE150 shLAMP2a and 510 shLAMP2a cell lines as compared to control cell lines. (e) Relative mRNA expression of LAMP2a detected by Q-PCR in LAMP2a overexpression cell lines and control cell lines. OE, LAMP2a overexpressed cells. \(^*p < 0.01\) compared with control cells. (f) Protein expression of LAMP2a detected by western blot in the indicated cell lines.
FIGURE 3  The activity of CMA influences ESCC cell proliferation. Growth curve of indicated cell lines by IncuCyte (a–d). **p < 0.01 LAMP2a modified cells compared with control cells. (e–h) Colony formation ability of the indicated cell lines. *p < 0.05,**p < 0.01 LAMP2a modified cells compared with control cells. (i) RND3 expression in indicated LAMP2a modified cells and comparative control cells by western blot.
CMA activity is required for rapid proliferation of ESCC cells

As more than 90% of esophageal cancers in China are squamous cell carcinoma and cell proliferation ability is an important biological characteristic of tumors, we detected the role of CMA activity in the proliferation of ESCC cells through regulating LAMP2a.

We analyzed the expression of LAMP2a in a panel of ESCC KYSE cell lines: 140, 150, 180, 270, 410, 450, and 510 by Q-PCR and western blot and found that LAMP2a was expressed in all of these cell lines but the expression level was distinct in different cell lines. We chose LAMP2a highly expressed KYSE 150, 510 cell lines to perform LAMP2a knockdown and lowly expressed KYSE 180, 410 cell lines to perform LAMP2a overexpression using lentiviral vector carrying shRNA specific to LAMP2a or LAMP2a cDNA, respectively. Through puromycin selection, we established cell lines 150shLAMP2a and 510shLAMP2a with stably reduced expression of the LAMP2a as detected by Q-PCR (Figure 2c) and western blot (Figure 2d). We also confirmed overexpression of LAMP2a in KYSE180 and 410 cell lines by Q-PCR (Figure 2e) and western blot (Figure 2f).

After that, we detected the proliferation capacity of the above-mentioned genetically modified ESCC cell lines in vitro by the Incucyte Live-Cell Analysis System. It was found that the growth rates of KYSE150shLAMP2a and
KYSE510shLAMP2a cell lines were decreased (Figure 3a,b), while overexpression of LAMP2a in KYSE180 and 410 cells did not increase cell growth rate of KYSE180 and 410 cell lines comparing to their counterpart cell lines (Figure 3c,d). The cell colony formation assay showed that knockdown of LAMP2a decreased the colony formation rate to more than 10% (Figure 3e,f), while overexpression of LAMP2a increased the colony formation rate to more than 10% as compared with the control cell lines (Figure 3g,h). These results indicate that the level of LAMP2a or CMA activity influences ESCC cell proliferation and transformation.

Previous research found that CMA regulates the proliferation of gastric cancer cells through RND3. LAMP2a silencing can increase the expression level of RND3. We detected the level of RND3 in these LAMP2a-modified ESCC cell lines by western blot but did not find any significant difference of RND3 expression in the LAMP2a knockdown or overexpression cell lines compared with their parental cell lines (Figure 3i). This result suggests that CMA regulates ESCC cell proliferation independent of RND3.

**Inhibition of CMA decreases cisplatin resistance of ESCC cells**

Cisplatin is widely used in clinics for chemotherapy of ESCC. Previous research showed that inhibition of macroautophagy can increase the sensitivity of ESCC cells to cisplatin as macroautophagy can wipe out the damaged proteins and thus inhibit cell death. There are cross-talks between macroautophagy and CMA. CMA was reported to influence chemoresistance in nonsmall cell

**FIGURE 5** Maximal activation of CMA increases cisplatin resistance in ESCC cell lines. (a, b) MTS detection of indicated cells with different concentrations of cisplatin treatment. Overexpression of LAMP2a in KYSE180 and 410 cells did not increase cisplatin resistance. (c, d) Cisplatin resistance increased in CMA maximal activated cell lines: 180 + CMA and 410 + CMA. *p < 0.05, **p < 0.01 CMA activated cells compared with control cells. (e) EIF4A1 expression level in indicated cells. As a substrate of CMA, EIF4A1 was digested more in CMA maximal activated cell lines than in LAMP2a overexpressed cell lines.
lungs. We therefore detected whether CMA regulates cisplatin resistance in ESCC cells. In a dose–response study, after treatment with various concentrations of cisplatin, the IC50 values of LAMP2a knockdown cell lines KYSE150 shLAMP2a and 510 shLAMP2a were decreased to half of their parental cell lines (Figure 4a,b) and knockdown cell lines showed reduced cell viability when treated by cisplatin as compared with their parental cell lines, which contributes to cisplatin sensitivity (Figure 4c,d).

Through long-term repeated low concentration of cisplatin treatment of the KYSE150 cell line, we got the cisplatin resistant cell line 510CDDP. We found the expression of LAMP2a is higher in the 510CDDP cell line compared with the parental 510 cell line (Figure 4e). We then knocked down LAMP2a in the 510CDDP cell line (Figure 4e) and found that the cisplatin resistance was partially lost in the 510CDDP shLAMP2a cell line as compared with the 510CDDP cell line, and the difference is significant (Figure 4f). All these results indicate that inhibition of CMA can decrease cisplatin resistance of ESCC cells.

**Maximal activation of CMA increases cisplatin resistance of ESCC cells**

We detected whether activating CMA can increase the cisplatin resistance in ESCC cell lines. Unexpectedly, the IC50 values of LAMP2a overexpressed ESCC cell lines were similar to those of their parental cell lines (Figure 5a,b), which means activating CMA through overexpression of LAMP2a cannot influence cisplatin resistance in ESCC cells. We speculated this maybe because the CMA activity level in LAMP2a overexpression cell lines is not high enough. We used small molecular compounds AC220 (inhibiting Flt3) and Spautin-1 (inhibiting macro-autophagy) to maximally activate CMA as previously reported and found the cisplatin resistance of ESCC cell lines was increased in this way. The IC50 value was about 3–4-fold higher in CMA activated cells than in control cells (Figure 5c,d). To compare the level of CMA activity between LAMP2a overexpressed cell lines and small molecular compound stimulated cell lines, we detected the level of eIF4A1 which was reported as a substrate of CMA in these cell lines. The activity of CMA as indicated by the level of eIF4A is indeed higher in AC220 and Spautin-1 stimulated cell lines than in LAMP2a overexpressed cell lines. These results suggest that highly activated CMA can influence cisplatin resistance in ESCC cells, but the CMA activity threshold that can contribute to cisplatin resistance still needs to be investigated further.

**Downregulation of LAMP2a improve the chemotherapy efficacy of cisplatin to ESCC in vivo**

We established ESCC xenograft mouse models on nude mice using KYSE 150 and 150shLAMP2a cell lines. We found that the tumor growth rate of 150 cells is higher than that of 150shLAMP2a cells in vivo (Figure 6), which was consistent with the result in vitro as shown in Figure 3a. To detect the cisplatin treatment efficacy in vivo, 4 mg/kg cisplatin was administered to tumor-bearing mice randomly 1 week after tumor cell implantation. The tumor growth was regressed in both groups but the cisplatin treatment efficacy in the 150shLAMP2a group was better than in the 150 group (p < 0.05). These results indicate that inhibition of CMA through targeting LAMP2a can enhance the treatment efficacy of cisplatin to ESCC.

**DISCUSSION**

Great efforts have been devoted to understanding the underlying mechanism of tumorigenesis and therapy resistance in order to identify new therapeutic targets for ESCC. In this study, we investigated the role of CMA in ESCC and found that down-regulating the activity of CMA can inhibit the proliferation and colony formation of ESCC cells and increase their sensitivity to cisplatin.

CMA can be activated in response to cellular stress and it targets cytosolic proteins carrying the KFERQ pentapeptide. This sequence was found in approximately 30% of cytosolic proteins. When the pentapeptide is exposed and recognized by Hsc70, the protein can be degraded by CMA in theory. CMA can therefore control many cellular processes in different physiological or pathological settings. Many cellular pathways that contribute to tumorigenesis were reported to be regulated by CMA.

ESCC clinical samples showed that highly malignant tumor tissues have high expression of LAMP2a, which
indicates high activity of CMA. In this paper, we detected whether and how the activity of CMA influences the tumorigenesis of ESCC. At first, we found LAMP2a-mediated CMA affects the proliferation of ESCC cells through knocking down or overexpressing LAMP2a. Recent study has shown that RND3 can be regulated by CMA and influences cancer cell proliferation in gastric cancer. However, we found no significant difference of RND3 in both LAMP2a knockdown and overexpressed cell lines compared with their counterpart cell lines (Figure 3i). This result suggests that RND3 is not the substrate of CMA in ESCC cell lines. The major targets of LAMP2a that influence cell proliferation in ESCC need to be further investigated.

Cisplatin, paclitaxel, and 5-fluorouracil are widely used chemotherapeutic drugs for ESCC in clinics, but drug resistances occur commonly and reduce their therapeutic efficacy. Previous research reported that many signaling pathways, such as AKT and NFκB, and some biological processes such as glycolysis, influence cisplatin resistance in ESCC. In this paper, we detected whether CMA influences the chemoresistance of ESCC too. ESCC cells with down-regulated LAMP2a were more sensitive to cisplatin through inhibiting apoptosis (Figure 4). Only maximal activation of CMA by AC220 and spautin-1 can further increase cisplatin resistance of ESCC while mild overexpression of LAMP2a cannot (Figure 5). This may be because overexpression of LAMP2a in the tested cell lines is not sufficient to digest substrates that influence cisplatin resistance. Bona fide substrates of CMA in regulating cisplatin resistance and the threshold of CMA activity that can cause cisplatin resistance need to be investigated further in the near future. Of note, AC220 and spautin-1 did not induce cell apoptosis in ESCC cell lines as reported in OCI-AML3 cells in previous research, but they did inhibit ESCC cell proliferation. Forty-eight hours later, changing the cell culture medium without AC220 and spautin-1, cell growth recovered (data not shown). As CMA activation also can increase cisplatin resistance, drugs that inhibit macroautophagy but activate CMA should be reconsidered to treat cisplatin-resistant tumors.

In summary, our results suggest that CMA can be a therapeutic target of ESCC.

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CONFLICT OF INTEREST
All authors declare no potential conflict of interests.

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