Regulatory Effects of Survivin on Autolysosome Formation-Related Proteins in Esophageal Carcinoma Cells

Hui Li

Xinjiang Medical University

LING LIU (✉ liulingpine@126.com)

Xinjiang Medical University <https://orcid.org/0000-0002-8688-2184>

Palidan Aiziti

Xinjiang Medical University

Shimao Zhu

Xinjiang Medical University

Shabahaiti Wusiman

Xinjiang Medical University

Yinyin Yang

Xinjiang Medical University

Yongchen Wang

Xinjiang Medical University

Hainisayimu Tuerxun

Xinjiang Medical University

Na Yi

Xinjiang Medical University

Huiwu Li

Shantou University

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Abstract

Purpose: To investigate the regulatory effect of Survivin on autolysosome formation-associated proteins in esophageal cancer cell (ESCC) line Eca109.

Methods: An autophagy induction model was constructed using rapamycin (RAPA), an autophagy inhibitor. The Survivin overexpression vector, shRNA vector, and Survivin inhibitor YM155 were used to interfere with the autophagy model. Laser copolymer microscopy technology was utilized to observe the changes in autophagosomes and their progression, while western blotting was used to detect the level of lysosome formation-related proteins after autophagy. Besides, immunofluorescence staining was used to determine the pH of lysosomes and the distribution of cathepsin-D in cells.

Results: (1) Eca109 cells transfected with MRFP-EGFP-LC3 vector by RAPA showed a significant increase in green fluorescence, indicating an increase in autophagosomes. Results from western blotting indicated a rise in protein expression levels of LC3-I and LC3-II, an increase in the ratio of LC3-II/LC3-I, and a decrease in the expression of p62 levels, indicating enhanced autophagy. (2) In the autophagy model of Eca109 cells, after Survivin overexpression, LC3 expression increased, ATG5 expression decreased ($P < 0.05$). After YM155 intervention, ATG5 and ATG16L1 expression rose ($P < 0.05$). (3) In the autophagy model of Eca109 cells, Rab7 and ATG5 expression increased, while ATG16L1 expression decreased ($P < 0.05$) after YM155 intervention and Survivin shRNA transfection. In the presence of the Survivin overexpression vector, YM155 interfered with the expression of ATG5, ATG16L1, and Rab7 in the autophagy model ($P < 0.05$). (4) Decreased Survivin can reduce p62 expression and increase autophagy. (5) Survivin overexpression increased the number of intracellular lysosomes and was involved in the regulation of lysosomal pH changes. Besides, inhibition of Survivin expression resulted in the extravasation of cathepsin-D, which was dispersive and affected the permeability of lysosomes.

Conclusion: Survivin expression was directly related to the expression of the autophagy-associated protein LC3 and P62. Survivin is associated with the autophagic process in cancer cells at specific periods, which indicates that Survivin is involved in both early autophagy formation and the later stages of autolysosomal function implementation.

Introduction

Autophagy is a commonly occurring survival mechanism in normal eukaryotic cells as well as diseased cells. It is based on balancing energy sources and coping with nutritional stress and plays an important role in maintaining the self-stable state of cells and creating conditions for cell survival[1]. Normally, autophagy in normal cells happens at a slow rate, but when cells lack nutrients, proteins accumulate and are induced to be upregulated under conditions of stress[2]. Based on the above environmental pressures, cells encapsulate intracellular substances, then utilize lysosomes to degrade self-damaged organelles and macromolecular substances under the regulation of autophagy related genes (ATG) and release small molecules to be recycled for cell physiological activities[3]. Because this promotes better cell

metabolism, autophagy is a biological process that takes place in normal organisms[4]. At the same time, autophagy also occurs in the process of many diseases, including the development and progress of cancer [5]. Moreover, autophagy can be considered as the "recycling factory" of cells. It also promotes energy efficiency by producing ATP and modulating damage control to remove functional proteins and organelles. Autophagy is a self-degradative process that is essential for balancing sources of energy at critical times during development and in response to nutrient stress[6]. It also serves the function of removing misfolded or aggregated proteins, eliminating intracellular pathogens, and clearing damaged organelles, such as mitochondria, endoplasmic reticulum, and peroxisomes. Thus, autophagy is generally regarded as a survival mechanism[7]. The basic process of autophagy includes induction of autophagy, production and maturation of autophagosomes, and binding of autophagosomes to lysosomes[8–12]. Autophagy is not only an essential cellular mechanism that plays a housekeeping role in normal physiological processes for error correction, but it is also involved in a variety of biological functions such as development, cellular differentiation, and the defense against pathogens and nutritional starvation[13]. The autophagy process is strictly regulated by autophagy-related genes, including LC3-I, LC3-II (microtubule-associated protein 1 light chain 3), ATG5 (autophagy related gene 5), ATG16L1 (autophagy related 16 like 1), Rab7(Ras-like in rat brain), and p62. Besides, the ratio of the LC3-II/I proteins can help to estimate the extent of autophagy [14, 15].

Autophagy can cooperate with apoptosis to promote cell death, and can also promote cell survival and antagonize apoptosis benefits. Rapamycin (RAPA) has been tested in clinical trials of cancer therapy and is believed to inhibit tumor growth by blocking protein translation and inducing autophagy[16–18]. In cancer, autophagy plays a dichotomous role by inhibiting tumor initiation but supporting tumor progression. The possible role of autophagy in tumor immunity means that the latest research into autophagy inhibitors has the potential to become a promising direction for tumor drug development [19].

Survivin is the strongest member of the inhibitor of apoptosis (IAP) protein family. The full length of the gene is 14.7 kb, it is located on chromosome 17q25, and it has the effect of regulating the cell cycle and inhibiting apoptosis[20]. Additionally, YM155 is a small molecule that can specifically inhibit the transcription activity of Survivin and directly inhibit Survivin expression to induce apoptosis [21]. Selective expression of Survivin in tumor tissue and its strong anti-apoptotic effect are regarded as new and important targets for the treatment of tumors. Several studies have shown that Survivin is specifically expressed in embryos, developing fetuses, and various forms of tumor tissue, while low or no expression is found in normal undifferentiated tissue [22]. Conversely, YM155 can induce the apoptosis of tumor cells, inhibit tumor cell growth, and enhance the sensitivity of tumor cells to radiation and chemotherapy by reducing the expression of Survivin. This has the potential to be an important direction in tumor gene therapy research [23].

Materials And Methods

1. Materials

We obtained ECA109 cell line from Cell Bank of Chinese Academy of Sciences;(cells have been identified), mRFP-EGFP-LC3 overexpression vector, Survivin overexpression vector, Survivin empty vector, Survivin shRNA vector from Hanheng Biology), rapamycin from Sigma company, YM155 from Selleck company. Anti-LC3I/II antibody, anti-ATG antibody, anti-p62 antibody, anti-Rab7 antibody, β -actin antibody, secondary anti-goat anti-rabbit IgG, goat anti-mouse IgG from Cell Signaling Technology.

2. Methods

2.1 Cell Culture

Human esophageal cancer cell line Eca109 was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, then was placed in a cell incubator at saturated humidity, 5% CO₂, and 37 °C for routine culture. The cycle of fluid change was 2 days. When the confluent rate of cell growth exceeded 85%, the passage was digested with 0.25% trypsin.

2.2 Cell Transfection

Eca109 cells in the logarithmic growth phase were injected into 6-well plates at a density of 1×10^5 cells per well when the degree of cell fusion reached 70%-80%.

After being washed and digested, the cells were lightly blown to form a single cell suspension and centrifuged at 1,200 rpm for 5 min at room temperature, then the supernatant was discarded. The cells were resuspended by adding an appropriate amount of electrotransfer buffer (usually 0.4 mL), and the suspended cells were again blown to make them homogeneous. They were then transferred into the electrotransformation cup, and the number of cells was regulated to be between $2.5-5 \times 10^6$ for each electrotransfer. Next, 5 μ g of plasmid was added, and the electrotransformation cup was placed into the sample tank for electrotransfer using a gene introducer. Three empty discharges were performed first, and the discharge time in the electrotransformation cup was usually kept in the range of 0.018 ~ 0.023. The optimal conditions for Eca109 cells are U: 290 V, C: 900 CF, R: 550 Ω , with a typical rotation time of 16–22 ms. A 6-well plate was prepared by adding 2 mL of complete medium to each well. Finally, after electrotransformation, we transferred the solution in the electrotransformation cup to the 6-well plate and placed it overnight in a 37°C incubator.

2.3 Western Blotting

The cells in the logarithmic growth phase were collected and treated accordingly. For each group, 200 μ l protein lysate was added to the cells and placed in an ice bath for 30 min to extract cell proteins. The cell deposition was discarded and the supernatant was collected after centrifugation at 13,000 r/min for 20 min at low temperature. Protein quantification was performed using a protein quantification kit. We performed SDS-PAGE on 6% concentrate gel and 12% separation gel with 40 μ g protein as the loading volume. The electrophoresis-separated protein was transferred to an NC membrane by the wet

electrotransfer method. Next, 5% skim milk powder was sealed at room temperature for 1.5 hours, and primary antibodies (LC3, ATG5, ATG16L1, p62, Rab7, β -actin, diluted to 1:1000) were added as usual, then incubated overnight at 4 °C. The following day, the membrane was heated to room temperature for 1 hour and washed with PBST three times, 5 minutes each time. Goat anti-rabbit secondary antibody (1:8000 dilution) was added and incubated at room temperature for 1.5 h. Then, the film was washed with PBST three times, 5 minutes each time. Finally, the solution was developed in a chemiluminescence instrument and scanned in grayscale, using β -actin as the internal reference. The results were statistically analyzed with the ratio of β -actin serving to determine protein levels.

2.4 Laser Confocal Microscope Observation

The mRFP-GFP-LC3 can simultaneously express red and green fluorescence. After transfection by mRFP-GFP-LC3, RAPA-induced Eca109 cells and control cells without RAPA-induced LC3 cells were taken and observed under a 400-fold field of vision. The number of cells expressing red and green fluorescence and the number of fluorescent particles in the cells expressing fluorescence were counted. Besides, the number of cells containing fluorescent particles was calculated.

2.5 Determination of Lysosomal Activity

After grouping and treatment of logarithmic phase growing cells, the liquid was removed 30 min before observation, and the complete medium containing 1 mg/mL LysoTracker Red probe was added for incubation in the dark for 30 min. The liquid was removed, washed with PBS three times, then the complete medium was added to observe the change of fluorescence. Lysosomes were fluorescence-labeled in red, LC-3 was labeled with green fluorescence, and the co-localization between the two was observed by merging the images.

2.6 Statistical Methods

We used SPSS Statistics 22.0 software for compiling data and conducting statistical analysis. The experimental data were expressed as mean $\pm$ SD and analyzed for the χ^2 test. A value of $P < 0.05$ indicated that the difference was statistically significant. All experiments were repeated three times.

Results

1. Establishment of Autophagy Model by RAPA Induction in Eca109 Cells

The number of LC3-expressing marked by mRFP-GFP increased in the RAPA-induced group ($P < 0.05$). Also, compared with the control group, the expression levels of LC3-I and LC3-II in the RAPA-induced group were substantially higher ($P < 0.05$), and the ratio of LC3-II/I increased. The expression of p62 protein was significantly lower than in the control group ($P < 0.05$). LC3-II is an important marker of autophagosomes and levels rise with an increase in autophagic membranes. During autophagy, p62 binds to the ubiquitinated protein to form a complex, which with the LC3-II protein localized on the inner

membrane of the autophagic vesicle, which is degraded together within the autophagic lysosome. Elevated LC3-II/I and reduced p62 levels indicate that autophagy is enhanced after RAPA induction, leading to the establishment of autophagy model cells (Fig. 1).

2. Effects of Survivin Overexpression and YM155 Inhibitor on Rab7, ATG5, ATG16L1, and LC3 in Eca109 Cells

After transfection with the Survivin overexpression vector, the expression of Survivin was significantly higher compared with the control group ($P < 0.05$). After YM155 was used to interfere with Eca109 cells, the endogenous expression of Survivin noticeably decreased ($P < 0.05$), indicating that YM155 is a functional inhibitor of Survivin and has a considerable effect on the inhibition of endogenous Survivin expression. This verifies that the Survivin low-expression cell model was successfully established (Fig. 2A).

Western blotting detection in the autophagy model showed that the protein expressions of LC3, ATG5, and ATG16L1 all increased ($P < 0.05$). While the expressions of Rab7 increased and Survivin decreased, it was not statistically significant ($P \geq 0.05$). After overexpression of Survivin in the autophagy model, there was an enhanced expression of Survivin and LC3 protein ($P < 0.05$), while the expression of ATG5 protein fell ($P < 0.05$). Moreover, the expression of Rab7 and ATG16L1 protein did not change appreciably. In the autophagy model, after the addition of YM155, the expression of Survivin, as well as the expression of ATG5 and ATG16L1, all decreased ($P < 0.05$), but LC3 and Rab7 only marginally increased ($P \geq 0.05$). After overexpression of Survivin in the autophagy model, the expression of Survivin protein did not significantly change after YM155 intervention, while the expression of Rab7, ATG5, and ATG16L1 protein all declined ($P < 0.05$). Besides, the expression of LC3 protein increased only slightly ($P \geq 0.05$). While the results are inconsistent, we can see that the expression of LC3, ATG5, and ATG16L1 all increased when we established the autophagy model. Survivin expression was directly related to the expression of the autophagy-associated protein LC3 (Fig. 2B).

3. Effects of Survivin-shRNA Intervention on ATG16L1, LC3, Rab7, and ATG5 in Eca109 Cells

In the RAPA autophagy model, levels of ATG16L1, LC3, Rab7, and ATG5 rose, while Survivin fell ($P < 0.05$). This indicates that Survivin can inhibit autophagy. After transfection with Survivin-shRNA in the autophagy model, the expression of the Survivin in the Survivin-shRNA group was lower, compared with the autophagy group ($P < 0.05$). This implies that Survivin-shRNA vector transfection was successful. At the same time, levels of LC3, Rab7, and ATG5 increased, suggesting that Survivin loss can increase autophagy. Compared with the RAPA group, the protein expressions of Rab7 and ATG5 in the Survivin-shRNA + RAPA group increased ($P < 0.05$), while the expression of ATG16L1 decreased ($P < 0.05$). This also confirms that Survivin can affect Rab7 and ATG5 expression during autophagy (Fig. 3).

4. Effect of Low Survivin Expression on p62 in Autophagy Model

After YM155 was added to interfere with the autophagy model, the expression level of p62 protein fell dramatically ($P < 0.05$), along with a decrease in p62 and increased autophagy levels (Fig. 4A,C). In the autophagy model (RAPA), the overexpression of Survivin under the interference of YM155 (to inhibit Survivin expression) leads to greatly decreased expression levels of the p62 protein ($P < 0.05$) (Fig. 4A,D). Additionally, the expression level of p62 reduced significantly after transfection with Survivin-shRNA ($P < 0.05$) (Fig. 4B,E), suggesting that decreased Survivin can reduce p62 expression and increase autophagy. This means that the expression of p62 is dependent on Survivin, and these two substances share a direct ratio.

5. Effects of Survivin on Lysosomes in Autophagy Models

Compared with the autophagy model, the number of intracellular red spots in the Survivin overexpression group drastically fell ($P < 0.05$). After inhibition of Survivin expression, the number of red spots in the cells increased (Fig. 5A), indicating that the presence of Survivin can inhibit autophagy and the number of lysosomes in the autophagy model.

Survivin overexpression in the autophagy model significantly reduced the yellow fluorescence intensity of the LysoSensor, while Survivin intervention in YM155 and Survivin-shRNA enhanced the yellow fluorescence intensity of the LysoSensor. This suggests that Survivin is involved in the regulation of pH changes in lysosomes (Fig. 5B).

6. Effect of Survivin on Membrane Permeability of Lysosomal Cells in Autophagy Model

In the autophagy model, Survivin overexpression significantly reduced the amount of red fluorescence. Compared with the Survivin overexpression group, Survivin-shRNA alone or YM55 intervention alone increased the amount of red fluorescence appreciably. Immunofluorescence of cathepsin-D was speckled, suggesting that low Survivin expression may protect lysosomal degradation by inhibiting the permeability of lysosomal membranes. Under normal conditions, the immunofluorescence staining of cathepsin-D is speckled, but when lysosomal membrane permeabilization occurs, the immunofluorescence of cathepsin-D is diffuse. In the autophagy model, Survivin overexpression significantly lowered the levels of cathepsin-D in cytoplasmic protein ($P < 0.05$). The number of activated types (34kD) of cathepsin-D also fell noticeably. This implies that overexpression of Survivin can inhibit the protein levels of cathepsin-D in lysosomes, thus affecting lysosomal degradation (Fig. 6).

Discussion

The autophagy process is strictly regulated by autophagy-related genes. Target of rapamycin (TOR) is a type of Ser/Thr protein kinase, which in turn belongs to the PIKK family[17]. Besides, mTOR(mammalian target of rapamycin) is a key component in the process of autophagy regulation, and inhibition of mTOR activity promotes autophagy. The LC3 protein is a homolog of ATG8, a ubiquitin-like protein, which can be divided into two subtypes, LC3-I and LC3-II, in cells. While LC3-I is located in the cytoplasm, LC3-II is situated in the autophagosome membrane. In the process of autophagy, LC3-I is modified to bind with

phosphatidylethanolamine(PE), subsequently forming LC3-II. The content of LC3-II aggregated on the membrane of the autophagosome is proportional to the number of autophagy vesicles[24]. Therefore, to a certain extent, LC3-II reflects autophagy activity, and changes in autophagy can be dynamically detected by quantifying the expression of LC3. In our study, Eca109 cells were induced by RAPA to establish an autophagy model, and the number of green and red fluorescent spots was observed by laser confocal microscope. As the expression of LC3-I and LC3-II rose, the ratio between LC3-II and LC3-I increased, while the expression of p62 fell. Thus, the autophagy model was established.

Autophagy related gene 5 (ATG5) plays a key role in the process of autophagy and is closely related to the occurrence and development of tumors[25]. Abnormal expression of ATG5 in prostate cancer suggests enhanced autophagy activity and increased autophagosome formation, which plays a crucial role in the development of prostate cancer[26]. The up-regulation of ATG5 and autophagy in prostate cancer cells and colon cancer cells can promote cell proliferation and migration[27]. In epithelial ovarian cancer, the expression of ATG5 decreases with the progression of pathological stages, and ATG5 and p53 collaborate in the development of epithelial ovarian cancer [28, 29]. The formation of autophagy vesicles requires a pair of necessary ubiquitin-like coupling systems, namely the ATG12-ATG5-ATG16L1 and ATG8 (LC3) phosphatidylethanolamine (LC3-PE) systems. However, in ATG16L1-deficient cells, autophagosome formation and protein degradation are seriously affected [30]. Many studies used the ATG16L1 silencing gene to block the binding of the ATG12-ATG5 complex with cell membranes, causing autophagy to weaken and hindering the formation of autophagosomes[31]. Therefore, ATG5 and ATG16L1 can be used as detection indexes in the autophagosome formation stage. Rab7 (Ras-related protein 7) is a member of the GTP-enzyme family. At present, more than 70 kinds of Rab7 family member proteins have been discovered. It is a polypeptide composed of 208 amino acid residues[32], located on the membrane of autophagosomes. It is essential for autolysosomal maturation, which can guide the transport of intracellular substances along microtubules. As an effective multi-functional regulator of autophagy and endocytosis, it is involved in the generation of autophagosomal lysosomes. It is also a key molecule in the signaling pathway network that regulates the fusion process of autophagosomal lysosomes, participates in the regulation of vesicular transport, and controls the aggregation and fusion of late vesicular endosomes/lysosomes[33]. The transport and localization of late autophagosomes depends on cholesterol, and the pathway is cholesterol-sensing Rab7 effector ORP1L [34]. Activated GTP-Rab7 can regulate several functions, such as autophagosome formation, autophagosome transport along microtubules, and exosome and autophagosome maturation. It can also regulate lysosomal biogenesis through interaction with effector molecules, and regulate membrane transport in the late endosomal-lysosomal system[35]. Studies have shown that its dysfunction is associated with the proliferation, metastasis, and invasion of gastric cancer and breast cancer[36, 37]. Some studies have also established that Rab7 is a negative regulator of the growth and invasion of prostate tumors[38]. Therefore, Rab7 can be selected to detect autophagy lysosome formation and tumor development. The ubiquitin-binding protein p62 (Sequestosome 1, SQSTM 1) is involved in cell signal transduction, oxidative stress, and autophagy. When autophagy progresses smoothly, p62 binds with the autophagosome membrane protein LC3-II to degrade together in the autophagosome. The lysosomal degradation of autophagy will

lead to a decrease in the level of p62, which is negatively correlated with the level of autophagy[39]. In our research, survivin expression did not directly regulate the expression of autophagy-related genes.

Lysosomal membrane permeability is marked by the entry of soluble enzyme components from the lysosomal cavity into the cytoplasm. Cathepsin-D, an endogenous aspartate protease that is an important component of lysosomes and is involved in various physiological processes and signaling pathways, has been identified as a clear cancer marker[40, 41]. During tumor invasion and metastasis, activation of cathepsin-D catalyzes the degradation of basement membrane and extracellular matrixes to facilitate the formation of pathways through which tumor cells can migrate and enter the vascular wall. Abnormal expression of cathepsin-D is closely associated with the invasion and metastasis of breast cancer[42] and ovarian cancer[43]. Cathepsin-D is also involved in autophagy, and activation of cathepsin-D in the autophagy lysosomal pathway requires an acidic lysosomal environment. Changes in pH significantly reduce protein degradation capacity[44] and can be used as a diagnostic indicator of autophagy activity. We found overexpression of Survivin can inhibit the protein levels of cathepsin-D in lysosomes, thus affecting lysosomal degradation.

Conclusion

In this study, an autophagy model of Eca109 was induced by RAPA. In the autophagy model, increased LC3 expression after Survivin overexpression increased autophagy activity. The number of lysosomes in cells was reduced and protein levels of cathepsin-D in lysosomes were inhibited, thereby affecting lysosomal degradation. Consequently, we ascertained that Survivin can promote autophagy, as a high expression in tumors interferes with lysosomal function. When Survivin was inhibited, the protein expressions of ATG5, ATG16L1, and p62 decreased, indicating that autophagy was impeded. Also, the number of lysosomes rose slightly, as low Survivin expression may protect the normal function of lysosomes by changing the acidic environment or membrane permeability. Survivin and RAPA-induced autophagy in tumor cells is a concomitant regulatory relationship of a specific time and specific target genes, which is involved in the early stage of autophagosome formation and the later stages of autophagosomal function implementation. The ATG5, ATG16L1, and p62 proteins may regulate Survivin in autophagy formation, thus promoting the initiation of autophagosomes.

Declarations

Acknowledgments

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Authors' Contributions

Ling Liu and Huiwu Li conceived the study and revised the manuscript; Palidan Aiziti performed the experiments and was responsible for the draft manuscript; Shimao Zhu Shabahaiti Wusiman, Yinyin

Yang performed the data analysis; Yongchen Wang, Hainisayimu Tuerxun, and Na Yi analyzed data and organized the figures; Ling Liu, Huiwu Li, and Hui Li confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Statement: All the results and figures in this paper are reliable and valid.

Conflicts of Interest

The authors declare no conflict of interest for the publication of this manuscript.

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Figures

Figure 1

Establishment of autophagy model in Eca109 cells

A: After transfection of Eca109 cells with the mRFP-eGFP-LC3 vector by RAPA induction, establish the autophagy model.

a:Green fluorescence of Control+mRFP-GFP-LC3(BC); b.Red fluorescence of Control+ mRFP-GFP-LC3; c.Superposition of green fluorescence and red fluorescence of Control+ mRFP-GFP-LC3; d. Green fluorescence of mRFP-GFP-LC3+RAPA; e. Red fluorescence of mRFP-GFP-LC3+RAPA; f. Superposition of green fluorescence and red fluorescence of mRFP-GFP-LC3+RAPA.

B: Western blotting of the protein expression of LC3-I, LC3-II,p62 in control and RAPA groups

Figure 2

Survivin, Rab7, ATG5, ATG16L1 and LC3 expression in Eca109 cells under different conditions

A:Ttransfecting with the Survivin overexpression vector, establish the Survivin overexpression cell model. After applying YM155 intervention to Eca109 cells, the expression of endogenous Survivin reduced.

1: Blank control;2: Survivin cDNA;3Survivin cDNA+RAPA;4:Survivin cDNA+YM155.

B: In the autophagy model, the expression ATG5, ATG16L1, and LC3 protein all increased. After overexpression of Survivin, the expression of Survivin increased, LC3 expression increased, and ATG5 expression decreased. The expression of Survivin decreased, while ATG5 and ATG16L1 decreased after YM155 cell intervention in the autophagy model. After overexpression of Survivin and then YM155

intervention, there was no significant change in the expression of Survivin , and the expression of Rab7, ATG5, and ATG16L1 decreased

1: Blank control; 2: RAPA; 3: YM155; 4: RAPA + YM155; 5: Negative control; 6: Survivin cDNA; 7: Survivin cDNA+RAPA; 8: Survivin cDNA+YM155; 9: Survivin cDNA+RAPA+YM155.

Figure 3

Effect of Survivin-shRNA intervention on ATG16L1, LC3, Rab7, and ATG5 in Eca109 cells

After transfection of Survivin-shRNA under the autophagy model, western blotting (A, B) showed Survivin expression. In Survivin-shRNA + RAPA group compared with the RAPA group, Rab7, ATG5 , ATG16L1 and LC3 expression changed (A, C-F).

1: Blank control; 2: RAPA; 3: Survivin-shRNA; 4: Survivin-shRNA+RAPA.

Figure 4

Effect of low Survivin expression on p62 in the cellular autophagy model

A: The p62 protein expression level fell significantly after the application of YM155 intervention in the autophagy model and Survivin + autophagy model (A, C, D).

1: Blank control; 2: RAPA; 3: YM155; 4: RAPA+YM155; 5: Negative control; 6: Survivin cDNA; 7: Survivin cDNA+RAPA; 8: Survivin cDNA+YM155; 9: Survivin cDNA+RAPA+YM155.

B: The p62 protein expression level decreased considerably after transfection of Survivin-shRNA in the autophagy mode (B, E).

1:Negative control; 2:RAPA; 3: Survivin-shRNA; 4:Survivin-shRNA+RAPA.

Figure 5

Effect of Survivin on the number of lysosomes in cells and pH value in the autophagy model

A: The number of red spots in the cells of the Survivin overexpression group reduced appreciably. After inhibiting Survivin expression (g), the number of red spots in the cells increased.

Note: The red fluorescent marker in the figure can localize lysosomes.

a: Blank control+mRFP-GFP-LC3; b: NC+mRFP-GFP-LC3; c: RAPA+ mRFP-GFP-LC3; d: Survivin cDNA+RAPA+mRFP-GFP-LC3; e: SurvivincDNA+RAPA+mRFP-GFP-LC3+YM155; f: Survivin-shRNA+mRFP-GFP-LC3; g: Survivin-shRNA+RAPA+mRFP-GFP-LC3.

B: LysoSensor yellow fluorescence intensity decreased significantly after overexpression of Survivin in the autophagy model, while LysoSensor yellow fluorescence intensity was enhanced after intervention with YM155 and Survivin-shRNA.

a: Blank control+ mRFP-GFP-LC3; b: RAPA+mRFP-GFP-LC3; c: Survivin cDNA +RAPA+mRFP-GFP-LC3; d: YM155+RAPA+mRFP-GFP-LC3; e: Survivin-shRNA +RAPA+mRFP-GFP-LC3.

Figure 6

Effect of Survivin on the level of cytosolic membrane permeabilization in the autophagy model

A: The RAPA group increased red fluorescence, Survivin overexpression reduced the amount of red fluorescence, and Survivin-shRNA or YM155 intervention alone increased the amount of red fluorescence,

compared to the Survivin overexpression group. The immunofluorescence of cathepsin-D was speckled.

B: The amount of cathepsin-D in cytoplasmic proteins decreased substantially after Survivin overexpression and the number of activated types of cathepsin-D fell significantly.

1: Negative control; 2: RAPA; 3: Survivin cDNA ; 4. Survivin cDNA+RAPA.