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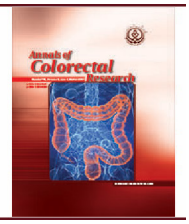

The Role of Endoplasmic Reticulum Metallo Protease 1 on Autophagy Pathway in HCT-116 Colorectal Cancer Cell Line

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Abstract

Background: Autophagy and the unfolded protein response (UPR) are mechanisms with dual roles in both maintaining cellular homeostasis and controlling the progression of various diseases such as cancer. Therefore, the identification of different molecules and proteins involved in the regulation of these pathways may contribute to finding new therapeutic targets. A member of the M28 family of the metallopeptidases, endoplasmic reticulum metalloprotease 1 (ERMP1), is overexpressed in cancers such as colorectal cancer. The role of this protein in UPR activation was previously reported in breast cancer. We aimed to evaluate the role of ERMP1 in the activation of autophagy and apoptosis in colorectal cancer.
Methods: *ERMP1* gene silencing was performed using specific small hairpin RNA (shRNA) in the HCT-116 colorectal cancer cell line. Then, autophagy-associated protein markers including Beclin 1, p62, and LC3II were evaluated via western blotting. The effect of ERMP1 knockdown on cellular apoptosis was also assessed by propidium iodide staining flow cytometry analysis. Statistical analysis was performed using SPSS software version 20.
Results: All three autophagy markers increased significantly in the *ERMP1*-silenced HCT116 cell lines compared with negative control cells ($P<0.05$). It seems that *ERMP1* silencing inhibits autophagy at the flux stage. However, *ERMP1* knockdown had no significant effect on HCT-116 apoptotic cell death ($P>0.05$).
Conclusion: The oncogenic protein, ERMP1, activates autophagy in the HCT-116 colorectal cancer cell line. Targeting *ERMP1* may be considered as a proper approach in colorectal cancer therapy. Further investigations are required to confirm these results.

Keywords: ERMP1, Colorectal cancer, Target therapy, Endoplasmic reticulum stress

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Introduction

The increasing incidence of colorectal cancer during the recent decades is a major concern. A wide range of genetic alterations, mechanisms, and signaling pathways are involved in the development of cancers like colorectal cancer (1, 2).

Autophagy is a self-degradative process, which is activated in eukaryotic cells in response to stress conditions like hypoxia and starvation. In the endoplasmic reticulum stress state, autophagy preserves the cells by maintaining protein and lipid homeostasis, mitochondria metabolism, and organelle recycling (3). During the autophagy process, a series of molecular events occur, leading to the formation of vesicles called autophagosomes containing defective organelles and proteins that have lost their structure. Eventually, the contents of the autophagosomes are degraded by autophagosome-lysosome fusion (4). Microtubule-associated protein 1 light chain 3B (LC3) is essential for the maturation of the autophagosome during autophagy. The autophagy-related protein 4 (ATG4) protease cleaves the C-terminal polypeptide of LC3 and generates LC3I. Then, covalent conjugation of LC3I to phosphatidylethanolamine (PE) by a complex of ATG proteins (ATG3, ATG7, ATG12-ATG5-ATG6L) generates LC3II. LC3II is recruited to the membrane of autophagosomes and autolysosomes and is degraded by lysosomal hydrolysis. Therefore, LC3II is a marker of autophagy activation (5). Also known as sequestosome-1 (SQSTM1), P62 is a classical substrate and receptor of autophagy. P62 connects to ubiquitinylated protein substrates through the C-terminal ubiquitin-associated domain (UBA) and also binds to LC3 through its LC3-interacting region (LIR). Finally, P62 promotes selective degradation of ubiquitinylated proteins. Therefore, p62 is known as a key indicator of autophagy flux (6, 7).

In addition to the autophagy, another signaling pathway named the unfolded protein response (UPR) is activated following endoplasmic reticulum (ER) stress conditions (8). The UPR pathway initially stops protein translation to maintain homeostasis and restore normal ER activity by activating a series of signaling pathways; it also contributes to proper protein folding by increasing the production of molecular chaperones. Continuous stimulation and prolonged stress of the ER leads the UPR and autophagy pathways toward apoptosis (9).

Autophagy is one of the mechanisms that, besides maintaining cellular homeostasis, can lead to the development and progression of pathological conditions such as cancer (10). The role of autophagy in cancer is quite complex and still under discussion. It seems that in the early stages of cancer formation, autophagy plays a role in inhibiting the tumor, but in the late stages, autophagy helps cancer cells to survive (11, 12). Autophagy is also responsible for the resistance of cancer cells to anti-cancer drugs (13, 14).

In comparison with normal cells, proliferation is increased and occurs more rapidly in cancer cells, and glycolytic metabolism is also altered. Therefore, cancer cells have more metabolic requirements and experience more stress, making their survival more dependent on autophagy (15). Various studies have shown that inhibition of autophagy can play an important role in treatment processes (16, 17).

Considering that autophagy has a dual role both in cell survival and cell death, identification of different pathways and enzymes involved in the regulation of autophagy may contribute to finding new therapeutic targets.

Endoplasmic reticulum metalloprotease 1 (ERMP1) is a member of the M28 family of metallopeptidases. The ERMP1 protein with 898 amino acids plays an essential role in normal ovarian histogenesis and is encoded by the *ERMP1* gene located on chromosome 9p24 (18, 19).

A recent study indicated the overexpression of *ERMP1* in cancers like ovary, breast, lung, and colorectal cancer (20). During ER stress, the enhancement of *ERMP1* expression activated the UPR and facilitated the survival of breast cancer cell lines. On the other hand, *ERMP1* gene silencing inhibited the proliferation, migration, and invasion of SK-BR-3 and MCF7 breast cancer cell lines (20).

Since in addition to UPR, the autophagy process is also activated during ER stress, we hypothesized that autophagy may be another pathway that is affected by *ERMP1* oncogene and leads to cancer development.

Based on our best knowledge, there is still no study on the relationship between ERMP1 and autophagy in colorectal cancer. This made us interested in designing this study to investigate this relationship and gain a better insight into the mechanisms involved in the development of colorectal cancer.

Materials and Methods

Cell Lines and Culture

Two cell lines, HEK293T and HCT116, were prepared from the Bonyakhteh Company (Bonyakhteh, Tehran, Iran). RPMI 1640 and DMEM media (Bio Idea, Tehran, Iran) supplemented with 10% fetal bovine serum, 1% penicillin, 2 mM glutamine, and 1% streptomycin were used for culturing HCT116 and HEK293T, respectively, in a humidified CO2 (5%) incubator at 37°C. The cells in the 3rd-4th passages were used for all experiments.

Production of Lentivirus Encoding ERMP1 shRNA or Scrambled shRNA

The shRNA against the *ERMP1* gene (oligo ID: TRCN0000336766) and scrambled shRNA (negative control) were separately cloned into a lentiviral plasmid (pLKO.1) (Addgene; cat. #10878). The sequence of *ERMP1* shRNA and scrambled shRNA were 5'-GGACTTTGCTCGGCGTTTATT-3' and 5'-CCTAAGGTTAAGTCGCCCTCG -3,

respectively. Stably transduced cells were selected with puromycin and plasmids and were purified from a single colony using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, no. #K0502).

Confirmation sequencing was performed using the following primers: 5′-TGGACTATCATATGCTTACCGTAAC -3′ and 5′-GTATGTCTGTTGCTATTATGTCTA-3′.

Then, HEK293T cells were cotransfected with the pLKO.1 plasmid containing *ERMP1* shRNA or scrambled shRNA and the second-generation packaging system, envelope protein-coding plasmid pMD2.G (Addgene plasmid #12259), and packaging construct pSPAX2 (Addgene plasmid #12260).

The standard polyethylenimine (PEI)-mediated method was applied for cotransfection (21). In brief, HEK293T cells with about 80% confluency were cotransfected with 1.5 µg pSPAX2, 0.5 µg pMD2.G, and 2 µg of the pLKO.1 vector encoding ERMP1 shRNA or scrambled shRNA. Then, the obtained lentiviruses were collected from the media after 48 hours (h) by centrifugation (1250 rpm for 5 minutes) before being aliquoted and stored at −80 °C. A Lentivirus-Associated p24 ELISA Kit (Takara Bio USA, no. # 632200) was applied for lentiviral titration.

shRNA Lentiviral Transduction and Real-time qPCR Assay

HCT116 cells (6.0×10^5) were seeded in 6-well plates. After 24 h, the solution containing either lentivirus expressing *ERMP1* shRNA or scrambled shRNA was added at the multiplicity of infection (MOI) of 5-50 with polybrene (8 µg/ml). After another 24 h, the transduction medium was aspirated and replaced with fresh complete medium and 1 µg/ml of puromycin dihydrochloride (Santa Cruz Biotechnology, no. # sc-108071) for selection. The culture media was removed and replaced with freshly prepared selective media every 2-3 days.

After 3 weeks, the *ERMP1* knockdown efficacy was assessed by quantitative real-time PCR in the remaining puromycin-resistant clones. In brief, RNA extraction from cultured cells was performed using the Tripure RNA isolation kit (Roche Applied Science, Germany). Then, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The primer sequences applied for assessing the *ERMP1* knockdown efficacy were ERMP1 F: 5′- TCTTTTGGCACTTCAGCAC-3′, ERMP1 R: 5′-CCCACCATCCACTAATACAAC-3′, GAPDH F: 5′-CGACCACTTTGTCAAGCTCA -3′, and GAPDH R: 5′- AGGGGTCTACATGGCAACTG -3′.

Western Blotting

The autophagy markers were evaluated by western blotting according to the protocol described previously. In brief, after 72 starvations without changing the media, cells were harvested and centrifuged for 5 min (1500 *g*). The obtained pellets were rinsed once with phosphate-buffered saline (PBS). Cells were lysed using sonication and the NP-40 lysis buffer method. The composition of NP-40 lysis buffer was 20 mM pH 7.5 Tris-HCl, 0.5 mM PMSF, 100µM β-glycerol 3-phosphate, 0.5% Nonidet P-40, and 0.5% protease inhibitor cocktail. After sonication (3 times, 5 pulses/second) and centrifugation (10.000 *g* at 4 °C for 8 min), the cell lysate supernatant containing the proteins was stored for further analysis at -80 °C. The Lowry method was applied for the evaluation of the protein concentration. Electrophoresis was performed on 10-15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel. The samples were prepared by boiling (100 °C for 5 min). Based on the type of proteins, about 10-20 µg of samples were loaded on the gel and electrophorized. The separated proteins were transferred to 0.2 µM nitrocellulose membranes (Bio-Rad; #1620112) for 2 hours at 100 volts using transfer buffer (500 nM glycine, 50 mM Tris-HCl, and 20% methanol). The membranes were blocked (overnight at 4 °C) with 5% skimmed milk in 1X Tris-buffered saline containing 0.01% Tween 20 (TBST). Then, the membranes were incubated with desired primary antibodies (Anti-Beclin1, Anti-P62, and Anti-LC3II) (Cell signaling Technology, USA) in 1% milk and 1X TBST (overnight at 4 °C). Afterward, the membranes were washed three times with 1X TBST for 20 min and were subsequently incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit IgG (whole molecule) conjugated with the peroxidase enzyme; Sigma-Aldrich, Germany) for 2 h at room temperature. The membranes were then washed again three times and incubated for 2-3 min with enhanced chemiluminescence (ECL) reagents (Abcam, USA).

Visualization of the blots was performed using the ChemiDoc MP imaging system (Bio-Rad, USA) and the intensities of the blots were measured using the Image Lab densitometry software after normalization to GAPDH.

Apoptosis Assay by Flow Cytometry

The Nicoletti method was applied to evaluate cellular apoptosis (22). In brief, transfected HCT-116 cells (scrambled or *ERMP1* shRNA) were cultured in 6-well plates and incubated for 72 h under starvation. Then, the cells were detached using EDTA and centrifuged at 1500 *g* for 5 min at 4 °C. After washing the pellets once with PBS, the cells were re-suspended in a hypotonic PI lysis buffer (0.1% Triton X-100, 1% sodium citrate, 0.5 mg/ml RNase A, and 40 µg/ml propidium iodide) before being incubated for 30 min at 37 °C. The nuclei of the dead cells were determined by flow cytometry as a sub-G1 population and were analyzed with FlowJo software.

Statistical Analysis

In order to analyze the data, SPSS software version

20 (SPSS Inc., Chicago, IL, USA) was used. The Mann–Whitney test, the Kruskal–Wallis test, and Bonferroni's post hoc test were conducted. Data representation was based on mean±standard deviation (SD). P-values less than 0.05 were considered statistically significant and all experiments were independently replicated three times.

Results

The Effect of ERMP1 Knockdown on Autophagy

The role of ERMP1 in the autophagy pathway was investigated by *ERMP1* silencing in the HCT116 cell line. For this purpose, the autophagy-associated protein markers including Becline-1, p62, and LC3βII/LC3βI were assessed and compared by western blotting in the *ERMP1*-shRNA treated cell line and the scrambled negative control shRNA-treated cells (Figure 1). The band intensity of the above markers was averaged from three independent repetitions of experiments (Figure 1A) and finally, the relative quantifications of Becline-1, p62, and LC3βII/LC3βI were determined (Figure 1 B, C, D). Our results indicated that all three autophagy markers increased significantly in the *ERMP1*-silenced HCT116 cell lines compared with negative control cells ($P<0.05$ for Becline-1 and LC3βII/LC3βI and $P<0.01$ for p62).

ERMP1 Knockdown Had no Significant Effect on Apoptosis

The results of the apoptosis assay revealed that although apoptosis slightly increased in *ERMP1*-knockdown HCT116 cells compared with negative controls (12.5% vs 11% Sub G1), the increase was insignificant ($P>0.05$) (Figure 2).

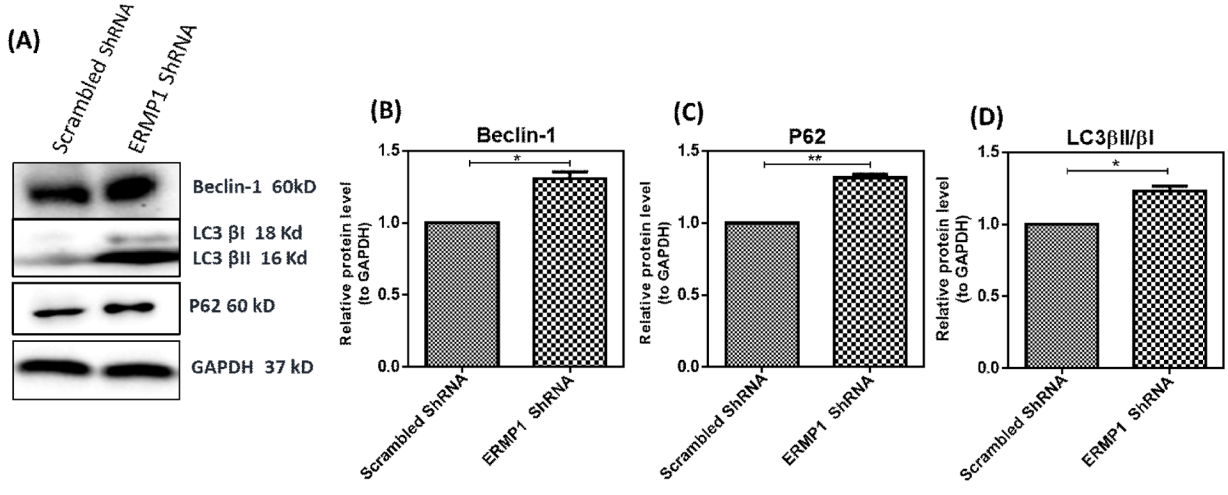

Figure 1: The effect of *ERMP1* knockdown on autophagy in HCT-116 cells. The expression of protein markers of autophagy (Beclin-1, P62, and LC3) was determined by western blotting. GAPDH was used as the loading control (A). *ERMP1* knockdown significantly increased the amount of the Beclin-1, P62, and LC3 proteins compared with the control (Scrambled shRNA) (B, C, D). Immunoblots are representative of three different biological replicates. (*P<0.05, **P<0.01).

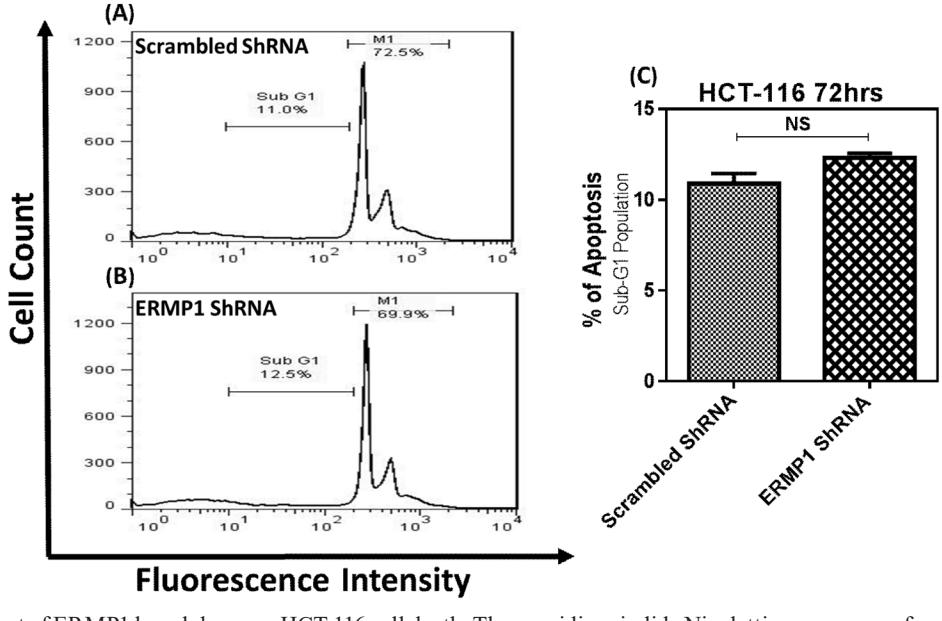

Figure 2: The effect of ERMP1 knockdown on HCT-116 cell death. The propidium iodide Nicoletti assay was performed for evaluation of cell death (A, B). *ERMP1* knockdown could not significantly induce apoptosis compared with the corresponding control (C). Data are presented as mean±SD of three replicates from three independent experiments. (NS, non-significant).

Discussion

The UPR and autophagy are two signaling pathways that are triggered during ER stress to maintain cellular homeostasis. For survival under starvation conditions, cancer cells are metabolically dependent on catabolic processes like autophagy. Therefore, recognizing the pathways and molecules involved in autophagy regulation may help us in discovering promising therapeutic targets (23).

There are limited studies on the ERMP1 protein. Qu et al. indicated that miR-148b downregulates *ERMP1* gene expression and suppresses the oxidative stress response in endometrial cancer cells, and therefore acts as a tumor suppressor (24).

In a study by Grandi et al., the role of ERMP1 in UPR activation was described (20). They evaluated the expression of the *ERMP1* gene in breast, ovary, lung, and colorectal cancer cell lines and found its over-expression in all above cancers irrespective of their grade and stage. They also reported the expression enhancement of the *ERMP1* gene during ER stress in breast cancer cell lines, which led to activation of the UPR and survival signals. On the other hand, knockdown of the *ERMP1* gene inhibited proliferation, migration, and invasion while inducing cell death in the SK-BR-3 and MCF7 breast cancer cell lines (20).

Considering the cross-talk between UPR and autophagy under ER stress conditions (25), we hypothesized that *ERMP1* over-expression may lead to cancer cells' survival through activation of the autophagy pathway in addition to the UPR pathway. To our best knowledge, there is still no study on the role of ERMP1 in autophagy activation in colorectal cancer cells. Therefore, this study was focused on this issue. Considering the high expression level of ERMP1 in the HCT116 cell line, this cell line was selected for our study. Our results indicated an increase of all three autophagy markers following *ERMP1* knockdown. Considering that Beclin-1 is an important factor in the initiation of autophagy (26), it seems that *ERMP1* silencing induces autophagy initiation. On the other hand, two other factors including p62 and LC3βII/LC3βI play key roles in the autophagy flux, and their levels decrease during the autophagy process (27). The increasing levels of these proteins in *ERMP1* knockdown cells demonstrated that *ERMP1* knockdown may inhibit autophagy at the flux stage.

Our finding also showed that *ERMP1* knockdown cannot significantly induce apoptosis in HCT116 cells.

Given that the overexpression of the *ERMP1* gene has been reported in various cancer cell lines such as ovary, breast, lung, and colorectal cancers, it seems that ERMP1 acts as an oncogenic protein (20). On the other hand, considering the results of previous studies and the present study, it can be inferred that ERMP1 has an important role in the activation of the UPR and autophagy signaling pathways in colorectal cancer cell lines (20). Since these signaling pathways lead to cancer cell survival, targeted inhibition of ERMP1 may lead to UPR and autophagy inhibition, eventuating in decreased survival in cancer cells.

Conclusion

ERMP1 is an oncogenic protein that activates the autophagy and UPR signaling pathways in colorectal cancer cell lines. Therefore, ERMP1 targeting may be a proper approach in colorectal cancer therapy. Further investigations would be helpful to confirm these results.

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Conflicts of interests: None declared.

References

1. Koveitypour Z, Panahi F, Vakilian M, Peymani M, Forootan FS, Esfahani MHN, et al. Signaling pathways involved in colorectal cancer progression. Cell & bioscience. 2019;9(1):1-14.
2. Amirghofran Z, Jalali SA, Ghaderi A, Hosseini SV. Genetic polymorphism in the transforming growth factor β1 gene (-509 C/T and-800 G/A) and colorectal cancer. Cancer genetics and cytogenetics. 2009;190(1):21-5.
3. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nature Reviews Cancer. 2007;7(12):961-7.
4. Yu L, Chen Y, Tooze SA. Autophagy pathway: cellular and molecular mechanisms. Autophagy. 2018;14(2):207-15.
5. Zhang Y, Zhang Y, Jin X-f, Zhou X-h, Dong X-h, Yu W-t, et al. The role of astragaloside IV against cerebral ischemia/reperfusion injury: suppression of apoptosis via promotion of P62-LC3-autophagy. Molecules. 2019;24(9):1838.
6. Lippai M, Lőw P. The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy. BioMed research international. 2014;2014.
7. Liu WJ, Ye L, Huang WF, Guo LJ, Xu ZG, Wu HL, et al. p62 links the autophagy pathway and the ubiqutin–proteasome system upon ubiquitinated protein degradation. Cellular & molecular biology letters. 2016;21(1):1-14.
8. Song S, Tan J, Miao Y, Zhang Q. Crosstalk of ER stress-mediated autophagy and ER-phagy: Involvement of UPR and the core autophagy machinery. Journal of cellular physiology. 2018;233(5):3867-74.
9. Suh DH, Kim M-K, Kim HS, Chung HH, Song YS. Unfolded protein response to autophagy as a promising druggable target for anticancer therapy. Annals of the New York Academy of Sciences. 2012;1271(1):20.
10. Xie W, Zhou J. Aberrant regulation of autophagy in mammalian diseases. Biology letters. 2018;14(1):20170540.

11. Singh SS, Vats S, Chia AY-Q, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. Oncogene. 2018;37(9):1142-58.
12. Choi KS. Autophagy and cancer. Experimental & molecular medicine. 2012;44(2):109-20.
13. Mele L, Del Vecchio V, Liccardo D, Prisco C, Schwerdtfeger M, Robinson N, et al. The role of autophagy in resistance to targeted therapies. Cancer Treatment Reviews. 2020;88.
14. Chen N, Karantza-Wadsworth V. Role and regulation of autophagy in cancer. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2009;1793(9):1516-23.
15. Amaravadi RK, Lippincott-Schwartz J, Yin X-M, Weiss WA, Takebe N, Timmer W, et al. Principles and current strategies for targeting autophagy for cancer treatment. Clinical cancer research. 2011;17(4):654-66.
16. Yang H-Z, Ma Y, Zhou Y, Xu L-M, Chen X-J, Ding W-B, et al. Autophagy contributes to the enrichment and survival of colorectal cancer stem cells under oxaliplatin treatment. Cancer letters. 2015;361(1):128-36.
17. Li J, Hou N, Faried A, Tsutsumi S, Kuwano H. Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. European journal of cancer. 2010;46(10):1900-9.
18. Garcia-Rudaz C, Luna F, Tapia V, Kerr B, Colgin L, Galimi F, et al. Fxna, a novel gene differentially expressed in the rat ovary at the time of folliculogenesis, is required for normal ovarian histogenesis. Development. 2007;134(5):945-57.
19. Wu J, Liu S, Liu G, Dombkowski A, Abrams J, Martin-Trevino R, et al. Identification and functional analysis of 9p24 amplified genes in human breast cancer. Oncogene. 2012;31(3):333-41.
20. Grandi A, Santi A, Campagnoli S, Parri M, De Camilli E, Song C, et al. ERMP1, a novel potential oncogene involved in UPR and oxidative stress defense, is highly expressed in human cancer. Oncotarget. 2016;7(39):63596.
21. Kuroda H, Kutner RH, Bazan NG, Reiser J. Simplified lentivirus vector production in protein-free media using polyethylenimine-mediated transfection. Journal of virological methods. 2009;157(2):113-21.
22. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods. 1991;139(2):271-9.
23. Chen X-l, Liu P, Zhu W-l, Lou L-g. DCZ5248, a novel dual inhibitor of Hsp90 and autophagy, exerts antitumor activity against colon cancer. Acta Pharmacologica Sinica. 2021;42(1):132-41.
24. Qu J, Zhang L, Li L, Su Y. MiR-148b functions as a tumor suppressor by targeting endoplasmic reticulum metallo protease 1 in human endometrial cancer cells. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics. 2018;27(1):81-8.
25. Deegan S, Koryga I, Glynn SA, Gupta S, Gorman AM, Samali A. A close connection between the PERK and IRE arms of the UPR and the transcriptional regulation of autophagy. Biochemical and biophysical research communications. 2015;456(1):305-11.
26. Antonioli M, Di Rienzo M, Piacentini M, Fimia GM. Emerging mechanisms in initiating and terminating autophagy. Trends in biochemical sciences. 2017;42(1):28-41.
27. Pugsley HR. Assessing autophagic flux by measuring LC3, p62, and LAMP1 co-localization using multispectral imaging flow cytometry. Journal of visualized experiments: JoVE. 2017(125).