Role of autophagy in cholangiocarcinoma: Pathophysiology and implications for therapy

Elisabetta Ninfole, Claudio Pinto, Antonio Benedetti, Marco Marzioni, Luca Maroni

Abstract

Cholangiocarcinoma (CCA) is a malignant tumour of the biliary system that originates from the neoplastic transformation of cholangiocytes. CCA is characterized by late diagnosis and poor outcome, with surgery considered as the last option for management. Autophagy is a physiological lysosomal degradation process, essential for cellular homeostasis and ubiquitous in all eukaryotic cells. Several studies have reported a potential involvement of autophagy in cancer, but it remains unclear whether activation of this process represents a survival mechanism of cancer cells. In the present review, we examine the autophagic process and summarize the current knowledge about the involvement of autophagy in the progression of cancer. The link between autophagy and chemoresistance and the use of autophagic markers in diagnosis are also considered in detail. Preliminary evidence shows that the combination of autophagy modulators (activators or inhibitors) with conventional chemotherapeutic agents offers a possible treatment option against signalling pathways that are hyperactivated or altered in CCA. In vitro evidence suggests that combination of chemotherapy agents, such as cisplatin, under activation or inhibition of autophagic processes, in two different CCA cell lines, may improve chemosensitivity and reduce cell survival, respectively. A deeper understanding of these pathways, in both cancer and non-cancer cells, could unveil possible therapeutic targets to treat CCA patients.

Key Words: Cholangiocarcinoma; Cholangiocytes; Autophagy; Apoptosis; Chemoresistance; Autophagolysosome

©The Author(s) 2021. Published by Baishideng Publishing Group Inc. All rights reserved.
INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumour that originates from the neoplastic transformation of cholangiocytes, the epithelial cells lining the biliary tree. CCA may arise at any point of the biliary tree, and according to the anatomical localization is commonly classified as intrahepatic (iCCA), perihilar CCA and distal CCA[1]. CCA represents the second most frequent type of primary liver neoplasms and, as for many other malignancies, incidence of CCA progressively increases with age. Several risk factors for CCA development have been identified. Among them, biliary infection with liver flukes (Ophistorchis viverrini and Clonorchis sinensis) is a dominant predisposing factor in Eastern countries, and it helps to explain the high prevalence of CCA in this region[2]. Compared to the general population, patients affected by primary sclerosing cholangitis have a 120-fold higher risk of developing CCA, which could arise early during the disease course[3]. Since CCA itself is often asymptomatic, it is generally diagnosed at an advanced stage. Radical surgical treatment remains the only curative treatment for CCA, but it is rarely possible due to disease extension. Thus, prognosis of patients remains dismal, with 5-year survival rates of only 15% to 30% for patients with a localized disease[4,5].

PHYSIOLOGICAL ROLES OF AUTOPHAGY

Autophagy is a physiological lysosomal degradation process, essential for cellular homeostasis and energy balance; as such, it is ubiquitous in all eukaryotic cells, being highly conserved from yeast to human. Yoshinori Ohsumi, winner of the 2016 Nobel Prize in Medicine, has contributed to extraordinary growth in this field through his studies in the yeast Saccharomyces cerevisiae. Takeshige et al[6] were indeed able to identify the “autophagic bodies”, and subsequently described the first autophagy-defective mutant, which then made it possible to identify several genes involved in autophagy in yeast[7]. After a few decades, it is now clear that in mammalian cells there are three primary types of autophagy, based on the mode of delivery of cargo into lysosomes: Microautophagy, macroautophagy and chaperone-mediated autophagy[8]. Macroautophagy (hereafter referred to as autophagy) is the most frequent and well-studied mechanism and occurs at basal rate under normal cellular homeostasis.

Autophagy is also activated following other stimuli, such as nutrient starvation, stress stimuli, hormonal stimulation, and pharmacological agents[9,10]. Autophagy starts with two major components that regulate induction/nucleation. During induction, pre-autophagosomal structures begin to create the membrane source, also known as the phagophore, which expands to engulf intra-cellular components. This step is regulated by the mammalian homologue of autophagy-related gene product 1 (Atg1), named Unc-51-like autophagy activating kinase (ULK1/2), which interacts with Atg13, Atg101 and the focal adhesion kinase-family interacting protein of 200 kDa. Nucleation of the phagophore occurs through the class III phosphatidylinositol 3-kinase (PI3K) Vps34, which recruits Beclin-1, p150/Vps15, and Ambr1. Beclin-1 is in turn inhibited by its binding to the anti-apoptotic protein BCL2. The elongation
mechanism of the membrane is regulated by the Atg5-Atg12 conjugation system and the Atg8/light chain 3 (LC3) conjugation systems that allow the formation of a double-membrane autophagosome. The mature autophagosome then merges with the lysosome, thereby promoting the degradation of the autophagosomal contents by acid proteases (formation of the autophagolysosome) (Figure 1).

In contrast, in microautophagy, cytosolic components are directly taken up by the lysosome itself, through invagination of the lysosomal membrane. Both macro- and microautophagy can engulf large structures with selective and non-selective mechanisms. Chaperone-mediated autophagy refers to the chaperone-dependent selection of soluble cytosolic proteins with a direct shuffling of these proteins across the lysosomal membrane, without additional vesicles. A detailed review of the autophagy apparatus has been provided elsewhere[11-13].

**METHODS FOR MONITORING AUTOPHagy**

Historically, evaluation of the autophagic process in experimental conditions has been challenging, due to the complex dynamic nature of the process itself. In fact, autophagy is a multi-step pathway that can be modulated at several levels, both positively and negatively. Therefore, the evaluation of the autophagic process by means of “static measurements” constitutes one of the first limits to such measures. A widely used marker is the microtubule-associated protein LC3, the mammalian homolog of yeast Atg8, which is a ubiquitin-like protein that is conjugated to phosphatidylethanolamine in the autophagosome formation. Accessible methods to monitor autophagy include fluorescence microscopy through conjugation of LC3 with green fluorescent protein or biochemical methods. Among the latter, the quantification of the conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound conjugated form) by immunoblotting or the LC3 turnover assay (degradation of LC3-II inside the lysosome estimated by comparison of two samples with and without lysosomal inhibitor treatment) represent two feasible and readily available methods to estimate autophagy activation[14]. In addition to LC3, autophagy can be evaluated by other markers, such as SQSTM1/p62 (sequestosome 1), an autophagy receptor that links ubiquitinated proteins to LC3 and accumulates in cells when autophagy is inhibited.

Among the methods to induce autophagy, starvation is the most potent physiological inducer, which exerts effects both *in vivo* and *in vitro*. The main pathway involved in nutrient signalling is the mammalian target of rapamycin (mTOR), which regulates cell growth and metabolism through phosphorylation of its downstream effectors. The mTOR protein kinase is composed of two complexes named mTORC1 and mTORC2, with the former being involved in nutrient homeostasis[15,16]. In the presence of nutrients, mTORC1 directly phosphorylates and inhibits the autophagy-initiating kinase ULK1. Therefore, inhibitors of the mTORC1 complex can induce autophagy[14] (Figure 2). The first generation of mTOR inhibitors was derived from rapamycin, the most famous mTOR inhibitor, initially widely used as an immunosuppressant drug in organ transplantation. These drugs include everolimus (RAD001), deforolimus (AP23573), and temsirolimus (CCI-779), followed by second-generation mTOR inhibitors, that include MLN0128 (sapanisertib), CC-233 or NVP-BEZ235 (dactolisib), and AZD-8055[17].

**INVolVEMENT OF AUTOPHagy IN CANCER**

The potential role of autophagy in cancer has been analysed in several studies. To date, it remains unclear however whether activation of the autophagic process favours or prevents the progression of tumour alterations.

*Nutritional deficiency/contribution*

Nutritional deficiency is a condition that characterizes several tumour microenvironments, including urinary bladder cancer. Human bladder cancer cells lines (T24 and 5637) treated with Hank’s balanced salt solution (commonly referred to as HBSS) show nutritional deficiency (starvation) that induces autophagy and promotes the invasion of bladder cancer cells. This mechanism is mediated by epithelial-mesenchymal transition (EMT), a process by which epithelial cells transform into mesenchymal cells through the transforming growth factor-beta 1 (TGF-β1)/Smad3 signalling pathway [18]. In a similar manner, starvation in HBSS induced autophagy in HepG2, a human...
Figure 1. The autophagyosome formation. Schematic representation of the pathways involved in the processes of induction/nucleation, elongation and final formation of the autophagyosome. LC3: Light chain 3.

Figure 2. Effects of induction/inhibition on autophagy. A: Autophagic flow and sites of action of inducers/inhibitors; B: Protein expression of light chain 3 (LC3) measured with the conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound conjugated form). LC3: Light chain 3; mTOR: Mammalian target of rapamycin.

Preliminary experimental data show an opposite influence of autophagy on pancreatic cancer. Among different risk factors that induce pancreatic cancer, diabetes provides a high glucose microenvironment that enables tumour growth by supplying high energy levels. Pancreatic cancer cell lines cultured in medium to imitate both normal and high blood glucose microenvironments showed a much higher prolif-
eration rate, suppressed apoptosis, inhibition of autophagy and enhanced sterol regulatory element-binding protein 1 (SREBP1) expression, when treated with high glucose. SREBP1, a transcription factor of lipid metabolism, is important in the malignant progression of many types of tumours and is inversely related with autophagy. Indeed, high glucose levels promote tumour growth in vivo by upregulating SREBP1 expression and by suppressing autophagy. In this case, stimulation of autophagy could prevent tumour growth in a high-glucose microenvironment[20].

**Autophagy inhibition/activation**

The PI3K/AKT/mTOR signalling pathway is hyperactivated or altered in many cancer types, including HCC, and regulates a broad range of cellular processes, including survival, proliferation, growth, metabolism, angiogenesis, and metastasis. mTORC1 inhibitors have been useful in understanding the role of autophagy in many cancers. In a study by Chang et al[21], NVP-BEZ235 was found to inhibit growth of two HCC cell lines: Hep3B and PLC/PRF/5. After treatment with NVP-BEZ235, induction of autophagy occurs in cell lines, accompanied by enhanced expression of LC3-II and decreased expression of p62. Moreover, inhibition of autophagy enhances the apoptotic pathway, suggesting that the combination of NVP-BEZ235 and autophagy inhibitors may be a strategy for the treatment of HCC, improving antitumor activity by enhancing apoptosis. NVP-BEZ235 has been also demonstrated to inhibit the PI3K/AKT/mTOR pathway and to induce autophagy in U266, KM3 and RPMI8226 cells, human multiple myeloma cells and multiple myeloma tumour-burdened nude mice[22]. AZD8055 inhibits cell proliferation and colony formation of the HCC cell lines Hep3B and Huh7 without induction of classical apoptosis evaluated by poly (ADP-ribose) polymerase (PARP) cleavage or caspase activation. In contrast, AZD8055 treatment was associated with induction of autophagy, which seems to be indispensable for the inhibitory effects of AZD8055[23].

As for autophagy activators, there is a recent growing interest for autophagy inhibitors, not only for research purposes but also as new therapeutic strategy. Commonly used pharmaceutical approaches to inhibit autophagy involve the use of PI3K inhibitors, such as wortmannin, LY294002 and 3-methyladenine, since class III PI3K activity is required for the autophagosome formation. Other pharmacological inhibitors block later stages of autophagy formation, such as ammonium chloride, bafilomycin A1, Lys05 and lysosomal protease inhibitors, such as E64D and pepstatin A, that preclude final degradation of autophagic cargo inside lysosomes[14]. Several clinical trials are evaluating the use of different autophagy inhibitors in the treatment of cancers; autophagy is indeed considered a survival mechanism of cancer cells, protecting them from apoptosis. The use of autophagy inhibitors could therefore improve the response to other agents[24]. For instance, PARP inhibitors have shown promising anticancer activity against ovarian cancers, and olaparib, rucaparib, and niraparib are the three PARP inhibitors approved by the Food and Drug Administration. Olaparib has been shown to induce autophagy in ovarian cancer cells, and autophagy provides an adaptive mechanism of PARP inhibitor resistance that can be overcome, as confirmed by inhibition of autophagy, with chloroquine, hydroxychloroquine, and LYS05. This could provide a new strategy for treatment with PARP inhibitors in combination with autophagy inhibitors[25].

**AUTOPHAGY IN CCA**

CCA remains a condition with limited therapeutic options. Surgery is the treatment of choice, but it is seldom performed since the disease is frequently diagnosed at advanced stages. Patients with unresectable or metastatic CCA are therefore candidates for chemotherapy. The therapeutic benefit of gemcitabine and cisplatin is considered the standard for patients with inoperable CCA and plays an established role in palliative care[26]. However, CCA responds very poorly to the current chemotherapy regimens[27,28]; the ineffectiveness of such is due to mechanisms of chemoresistance, characterized by a multidrug resistance phenotype that allows cancer cells to escape from the action of drugs[29]. The low sensitivity of CCA cells to antineoplastic drugs has been demonstrated extensively in vitro. Treatment with cisplatin for 12 h or 24 h resulted in a different response between HepG2 cells (human HCC cell line) and QBC939 (CCA cell line), since the latter were not affected by the action of the drug[30]. This difference could be due to stronger antioxidant ability in QBC939 cells, a mechanism correlated with autophagy. Autophagy inhibitors such as chloroquine and 3-methyladenine, combined with cisplatin or not, significantly inhibit
QBC939 cell viability. This effect was possibly correlated with an increased sensitivity of lysosome inhibition, thus suggesting a potential anticancer role[30]. Furthermore, chloroquine reduced the viability of QBC939 cells, due to the accumulation of large quantities of protein in the cytoplasm leading to endoplasmic reticulum stress and cellular apoptosis[31].

ABC294640 is a novel sphingosine kinase 2 (Sphk2) inhibitor, a lipid kinase with oncogenic role in cancer. Pharmacological inhibition of Sphk2 by ABC294640 inhibits CCA cell growth and induced caspase-dependent apoptosis. Furthermore, ABC294640 also induces autophagy and treatment with autophagy inhibitors potentiated ABC294640-induced cytotoxicity and apoptosis. Combination of ABC294640 with sorafenib was shown to synergistically inhibit cell proliferation of CCA cells, suggesting that ABC294640 with sorafenib and/or autophagy inhibitors could be a strategy for treatment of CCA[32]. A possible correlation between autophagy and antioxidant capacity has been highlighted recently, in another study. Incubations with ABT737, an inhibitor of the Bcl-2 family, combined with cisplatin, induced autophagy in RBE cells, another human CCA line, as demonstrated by an increase in LC3II/LC3I ratio at 12 h and 20 h. Overexpression of Bcl-2 family proteins is involved in the resistance to cisplatin[33].

DHA is an anti-malarial drug that induces cell death in CCA cell lines, both by caspase-dependent and caspase-independent cell death. Transcriptomic analysis in KUU-213 cells showed that both apoptotic genes and autophagy genes were induced, with downregulation of the anti-apoptotic BCL-2 gene and AKT-mTOR signalling pathway that inhibits autophagy. DHA also induced the expression of DAPK1, a pro-apoptotic gene that is associated with autophagy, as confirmed by increased conversion of LC3-I into LC3-II[34]. Inhibition of autophagic flux may be a possible therapeutic approach, as demonstrated by treatment with salinomycin, the efficacy of which for suppressing tumour cell viability was analysed in vitro and in vivo in CCA [35]. Sorafenib is a multikinase inhibitor drug, which is approved for first-line management of advanced HCC but has no effect on CCA. Recent research indicates that autophagy is induced under sorafenib stress and promotes the ability of sorafenib to kill HCC cells. Although the role of autophagy in sorafenib-treated HCC patients remains unclear, additional studies with the use of autophagy inhibitors may provide important clues on a possible role as adjuvant antineoplastic therapy[36]. Sorafenib treatment showed a differential effect on human HCC cell lines and human iCCA cell lines. This effect could be due to an escape from inhibition of the RAF/MEK/ERK pathway, with activation of the AKT/mTOR signalling cascade, through an increased phosphorylation of AKT Ser473.

As mentioned before, mTORC1 promotes cell growth by suppressing protein catabolism, most notably autophagy, while mTORC2 is not sensitive to nutrients and is mainly involved in the regulation of cell migration and cytoskeletal remodelling [15]. Following treatment with sorafenib, a dose-dependent activation of mTORC2, that is upstream of the AKT Ser473 pathway, was observed by western blot analysis in a human iCCA cell line, without involvement of mTORC1. Western blot analysis of LC3-II/total LC3 expression, following treatment with sorafenib, showed no involvement of autophagy. Altogether, sorafenib enhances mTORC2 and AKT Ser473 phosphorylation, with a reduction of FOXO1 expression, which leads to inhibition of apoptosis and facilitated cell survival in CCA cells[37]. The FoxO family members are transcription factors involved in metabolic homeostasis, neurogenesis and neuroprotection, skeletal muscle homeostasis and cardiac remodelling, with a debated involvement in autophagy[38]. Post-translational modifications can promote or prevent the activity of FoxO proteins by mediating their nuclear translocation or exclusion with the promoter regions to induce the expression of autophagy. FoxOs could also interact with autophagy proteins independently. In fact, following stress conditions (e.g., starvation or oxidative stress), the dissociation of deacetylases Sirt1 or Sirt2 from their substrate FoxO1 allows FoxO1 acetylation that is required for binding to Atg7 and stimulation of autophagy[38]. QBC939 cells subjected to serum starvation responded with acetylation of FOXO1 (AcFOXO1) and subsequent interaction with Atg7, leading to autophagy activation. Moreover, knockdown of FOXO1 or treatment with resveratrol, a Sirt1 agonist, inhibited the autophagic flux, resulting in oxidative stress, mitochondrial dysfunction, and apoptosis. These effects were mitigated by the action of rapamycin, an inhibitor of mTOR[39].

Correlation between autophagy/apoptosis and CCA cells has also been evaluated in a condition of amino acids deprivation, which mimics the lack of nutrition and oxygen typical of the tumour microenvironment. This state supports the induction of autophagy and the expression of its associated proteins, such as LC3-II and Beclin-1. Beclin-1 is a crucial regulator of autophagy and has been found deregulated in several
Autophagy in cholangiocarcinoma

Table 1 This table summarizes the effects of autophagy modulators on cholangiocarcinoma development

| Autophagy modulators                              | Effects on CCA                                      | Ref.                          |
|---------------------------------------------------|----------------------------------------------------|-------------------------------|
| **Inhibitors**                                    |                                                    |                               |
| Cisplatin combined with CQ/3-MA                   | ↓ Cell survival                                    | Qu et al [30], 2017           |
| CQ                                                | ↓ Cell survival                                    | Jia et al [31], 2018          |
| ABC294640 with sorafenib and autophagy inhibitors | ↓ Cell survival                                    | Ding et al [32], 2016         |
| Salinomycin                                       | ↓ Cell survival                                    | Klose et al [35], 2018        |
| Resveratrol                                        | ↑ Oxidative stress                                 | He et al [39], 2018           |
| **Activators**                                    |                                                    |                               |
| ABT737 combined with cisplatin                    | ↓ Chemoresistance                                  | Fan et al [33], 2015          |
| DHA                                               | ↑ Apoptosis                                         | Thongchot et al [34], 2018    |
| NVP-BEZ235                                        | No effect on apoptosis                             | Yothaisong et al [45], 2013   |
| Resveratrol                                       | ↓ IL-6                                             | Thongchot et al [46], 2018    |

CCA: Cholangiocarcinoma; IL: Interleukin; ER: Endoplasmic reticulum.

Table 1: This table summarizes the effects of autophagy modulators on cholangiocarcinoma development.

- **Inhibitors**
  - Cisplatin combined with CQ/3-MA: ↓ Cell survival (Qu et al [30], 2017)
  - CQ: ↓ Cell survival (Jia et al [31], 2018)
  - ABC294640 with sorafenib and autophagy inhibitors: ↓ Cell survival (Ding et al [32], 2016)
  - Salinomycin: ↓ Cell survival (Klose et al [35], 2018)
  - Resveratrol: ↑ Oxidative stress (He et al [39], 2018)

- **Activators**
  - ABT737 combined with cisplatin: ↓ Chemoresistance (Fan et al [33], 2015)
  - DHA: ↑ Apoptosis (Thongchot et al [34], 2018)
  - NVP-BEZ235: No effect on apoptosis (Yothaisong et al [45], 2013)
  - Resveratrol: ↓ IL-6 (Thongchot et al [46], 2018)

MicroRNAs (miRNAs) are noncoding small RNA molecules, single-stranded with a length of 18-25 nucleotides, that regulate fundamental cellular processes, including cell proliferation, metabolism and apoptosis, and are often unstable in cancer. MiRNAs play a crucial role in the development and progression of CCA. aberrant expression of miRNAs is involved in modulating the response to chemotherapy; moreover, some miRNAs can act as oncomiRs or tumour suppressor miRNAs, based on their targeted genes[47]. MiR-124, in particular, was found to be upregulated in the tumour tissue of patients and in CCA cell lines, and to exert a tumour suppressive function by inducing autophagy-related cell death via direct targeting of the EZH2-STAT3 signalling axis. Confirming this, overexpression of miR-124 upregulated the expression of Beclin-1 and the conversion from LC3-I to LC3-II with autophagy induction, together with downregulation of the anti-apoptotic factor BCL-2, which...
interacts with Beclin-1 in CCA cells[48]. In addition, miR-373 is reduced in CCA tissues as well as cell lines and suppresses autophagy by modulating ULK1 directly, leading to apoptosis[49].

CONCLUSION

Autophagy is an orchestrated multistep catabolic process, which allows cells to recover homeostasis under stressful conditions by controlling energy and nutrient balance. In CCA, several pieces of evidence strongly suggest a correlation with autophagy. The use of autophagy modulators (inductors or inhibitors) combined with pharmacological agents seems a very promising strategy to treat different cancers, thereby enhancing apoptosis by endoplasmic reticulum stress or due to mitochondrial dynamics. The clinical relevance of these findings and the breadth of any potential use of autophagy modulators in patients should be further developed. Nutritional deficiency is a condition of some tumours, characterized by microenvironments that lack nutrients and are poorly vascularised, and the role of autophagy in this condition needs to be clarified.

REFERENCES

1. Kendall T, Verheij J, Gaudio E, Evert M, Guido M, Goepbert B, Carpino G. Anatomical, histomorphological and molecular classification of cholangiocarcinoma. Liver Int 2019; 39 Suppl 1: 7-18 [PMID: 30852996 DOI: 10.1002/jcb.27788]
2. Potter JE, Stevenson AJ, Coleman-Minahan K, Hopkins K, White K, Baum SE, Grossman D. Challenging unintended pregnancy as an indicator of reproductive autonomy. Contraception 2019; 100: 1-4 [PMID: 30851238 DOI: 10.1016/j.contraception.2019.02.005]
3. Song J, Li Y, Bowlus CL, Yang G, Leung PSC, Gershwin ME. Cholangiocarcinoma in Patients with Primary Sclerosing Cholangitis (PSC): a Comprehensive Review. Clin Rev Allergy Immunol 2020; 58: 134-149 [PMID: 31463807 DOI: 10.1007/s12016-019-08764-7]
4. Blechacz B, Gores GJ. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. Hepatology 2008; 48: 308-321 [PMID: 18536057 DOI: 10.1002/hep.22310]
5. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 2013; 145: 1215-1229 [PMID: 24140396 DOI: 10.1053/j.gastro.2013.10.013]
6. Takeshige K, Baba M, Tsutui S, Noda T, Ohsumi Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol 1992; 119: 301-311 [PMID: 1400575 DOI: 10.1083/jcb.119.2.301]
7. Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS Lett 1993; 333: 169-174 [PMID: 8224160 DOI: 10.1016/0014-5793(93)80398-e]
8. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. Antioxid Redox Signal 2014; 20: 460-473 [PMID: 23725295 DOI: 10.1089/ars.2013.5371]
9. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. Mol Cell 2010; 40: 280-293 [PMID: 20965422 DOI: 10.1016/j.molcel.2010.09.023]
10. Zhan L, Li J, Wei B. Autophagy therapeutics: preclinical basis and initial clinical studies. Cancer Chemother Pharmacol 2018; 82: 923-934 [PMID: 30225602 DOI: 10.1007/s00270-018-3688-3]
11. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Res 2014; 24: 24-41 [PMID: 24366339 DOI: 10.1038/cr.2013.168]
12. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 2010; 221: 3-12 [PMID: 20225336 DOI: 10.1002/path.2697]
13. Ravanat P, Srikumar IF, Talwar P. Autophagy: The spotlight for cellular stress responses. Life Sci 2017; 188: 53-67 [PMID: 28866100 DOI: 10.1016/j.lfs.2017.08.029]
14. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010; 140: 313-326 [PMID: 20144757 DOI: 10.1016/j.cell.2010.01.028]
15. Kim J, Guan KL. mTOR as a central hub of nutrient signalling and cell growth. Nat Cell Biol 2019; 21: 63-71 [PMID: 30602761 DOI: 10.1038/s41556-018-0205-1]
16. Rabanal-Ruiz Y, Korolchuk VI. mTORC1 and Nutrient Homeostasis: The Central Role of the Lyssosome. Int J Mol Sci 2018; 19 [PMID: 29534520 DOI: 10.3390/ijms19030818]
17. Lu X, Paliogiannis P, Calvisi DF, Chen X. Role of the Mammalian Target of Rapamycin Pathway in Liver Cancer: From Molecular Genetics to Targeted Therapies. Hepatology 2021; 73 Suppl 1: 49-61 [PMID: 32394479 DOI: 10.1002/hep.31510]
18. Tong H, Yin H, Hossain MA, Wang Y, Wu F, Dong X, Gao S, Zhan K, He W. Starvation-induced autophagy promotes the invasion and migration of human bladder cancer cells via TGF-β1/Smad3-mediated epithelial-mesenchymal transition activation. J Cell Biochem 2019; 120: 5118-5127 [PMID: 30320898 DOI: 10.1002/jcb.27785]
Ninfole E et al. Autophagy in cholangiocarcinoma

19 Li J, Yang B, Zhou Q, Wu Y, Shang D, Guo Y, Song Z, Zheng Q, Xiong J. Autophagy promotes hepatocellular carcinoma cell invasion through activation of epithelial-mesenchymal transition. *Carcinogenesis* 2013; 34: 1343-1351 [PMID: 23430956 DOI: 10.1093/carcin/bg3063]

20 Zhou C, Qian W, Li J, Ma J, Chen X, Jiang Z, Cheng L, Duan W, Wang Z, Wu Z, Ma Q, Li X. High glucose microenvironment accelerates tumor growth via SREBP1-autophagy axis in pancreatic cancer. *J Exp Clin Cancer Res* 2019; 38: 302 [PMID: 31296258 DOI: 10.1186/s13046-019-1288-7]

21 Chang Z, Shi G, Jin J, Guo H, Guo X, Luo F, Song Y, Jia X. Dual PI3K/mTOR inhibitor NVP-BEZ235-induced apoptosis of hepatocellular carcinoma cell lines is enhanced by inhibitors of autophagy. *Int J Mol Med* 2013; 31: 1449-1456 [PMID: 23588698 DOI: 10.3892/ijmm.2013.1351]

22 Ma Y, Jin Z, Yu K, Liu Q. NVP-BEZ235-induced autophagy as a potential therapeutic approach for multiple myeloma. *Am J Transl Res* 2019; 11: 87-105 [PMID: 30787971]

23 Hu M, Huang H, Zhao R, Li P, Li M, Miao H, Chen N, Chen M. AZD8055 induces cell death associated with autophagy and activation of AMPK in hepatocellular carcinoma. *Oncol Rep* 2014; 31: 649-656 [PMID: 24297300 DOI: 10.3892/or.2013.2890]

24 Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat Rev Cancer* 2017; 17: 528-542 [PMID: 28751651 DOI: 10.1038/nrc.2017.53]

25 Santiago-O’Farrill JM, Weroja SJ, Hou X, Oberg AL, Heinzen EP, Maurer MJ, Pang L, Rask P, Amaravadi RK, Becker SE, Romero I, Rubio MJ, Matias-Guiu X, Santacana M, Llombart-Cussac A, Poveda A, Lu Z, Bast RC Jr. Poly(adenosine diphosphate ribose) polymerase inhibitors induce autophagy-mediated drug resistance in ovarian cancer xenografts, and patient-derived xenograft models. *Cancer* 2020; 126: 894-907 [PMID: 31714594 DOI: 10.1002/cncr.32600]

26 Vogel A, Wege H, Caca K, Nasbun B, Neumann U. The diagnosis and treatment of cholangiocarcinoma. *Disch Arztebl Int* 2014; 111: 748-754 [PMID: 25412631 DOI: 10.3238/arztebl.2014.0748]

27 Drayton RM, Catto JW. Molecular mechanisms of cisplatin resistance in bladder cancer. *Expert Rev Anticancer Ther* 2012; 12: 271-281 [PMID: 22316374 DOI: 10.1586/era.11.201]

28 Galluzzi L, Vitale I, Michels J, Brenner C, Szabadkai G, Harel-Bellan A, Castedo M, Kroemer G. Systems biology of cisplatin resistance: past, present and future. *Cell Death Dis* 2014; 5: e1257 [PMID: 24874729 DOI: 10.1038/cddis.2013.428]

29 Marin J, Lozano E, Herraez E, Asensio M, Di Giacomo S, Romero MR, Briz O, Serrano MA, Efferth T, Macias RIR. Chemoresistance and chemosensitization in cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* 2018; 1864: 1444-1453 [PMID: 28660147 DOI: 10.1016/j.bbadis.2017.06.005]

30 Qu X, Sheng J, Shen L, Su J, Xu X, Qie Q, Wu Y, Zhang X, Sun L. Autophagy inhibitor chloroquine increases sensitivity to cisplatin in QBC939 cholangiocarcinoma cells by mitochondrial ROS. *PLoS One* 2017; 12: e0173712 [PMID: 28301876 DOI: 10.1371/journal.pone.0173712]

31 Jia B, Xue Y, Yan X, Li J, Wu Y, Guo R, Zhang J, Zhang L, Li Y, Liu Y, Sun L. Autophagy inhibitor chloroquine induces apoptosis of cholangiocarcinoma cells via endoplasmic reticulum stress. *Oncol Lett* 2018; 16: 3509-3516 [PMID: 30127955 DOI: 10.3892/ol.2018.9131]

32 Ding X, Chaiteerakij R, Moser CD, Shaleh H, Boakye J, Chen G, Ndzengue A, Li Y, Zhou Y, Huang S, Sinicrope FA, Zou X, Thomas MB, Smith CD, Roberts LR. Antitumor effect of the novel sphingosine kinase 2 inhibitor ABC294640 is enhanced by inhibition of autophagy and by sorafenib in human cholangiocarcinoma cells. *Oncotarget* 2016; 7: 20800-20802 [PMID: 26950050 DOI: 10.18632/oncotarget.7914]

33 Fan Z, Yu H, Cui N, Kong X, Liu X, Chang Y, Wu Y, Sun L, Wang G. ABT737 enhances cholangiocarcinoma sensitivity to cisplatin through regulation of mitochondrial dynamics. *Exp Cell Res* 2015; 335: 65-81 [PMID: 25936772 DOI: 10.1016/j.yexcr.2015.04.016]

34 Thongchot S, Vidoni C, Ferraresi A, Loilome W, Yongvanit P, Namwat N, Isidoro C. Dihydroartemisinin induces apoptosis and autophagy-dependent cell death in cholangiocarcinoma through a DAPK1-BECLIN1 pathway. *Mol Carcinog* 2018; 57: 1735-1750 [PMID: 30136419 DOI: 10.1002/mc.22893]

35 Klose J, Guerlevik E, Trostel T, Kühnel F, Schmidt T, Schneider M, Ulrich A. Salinomycin inhibits apoptosis via mitochondrial dynamics. *Oncol Rep* 2018; 39: 843-850 [PMID: 29251327 DOI: 10.3892/or.2017.6153]

36 Heqing Y, Bin L, Xuehai Y, Linfa L. The role and mechanism of autophagy in sorafenib targeted cancer therapy. *Crit Rev Oncol Hematol* 2016; 100: 137-140 [PMID: 26920575 DOI: 10.1016/j.critrevonc.2016.02.006]

37 Yokoi K, Kobayashi A, Motomura H, Kitazawa M, Shimizu A, Notake T, Yokoyama T, Matsumura T, Takeoka M, Miyagawa SI. Survival pathway of cholangiocarcinoma via AKT/mTOR signaling to escape RAF/MEK/ERK pathway inhibition by sorafenib. *Oncol Rep* 2018; 39: 843-850 [PMID: 29251327 DOI: 10.3892/or.2017.6153]

38 Cheng Z. The FoxO-Autophagy Axis in Health and Disease. *Trends Endocrinol Metab* 2019; 30: 658-671 [PMID: 31443842 DOI: 10.1016/j.tem.2019.07.009]

39 He W, Zhang A, Qi L, Na C, Jiang R, Fan Z, Chen J. FOXO1, a Potential Therapeutic Target, Regulates Autophagic Flux, Oxidative Stress, Mitochondrial Dysfunction, and Apoptosis in Human Cholangiocarcinoma QBC939 Cells. *Cell Physiol Biochem* 2018; 45: 1506-1514 [PMID: 29466794 DOI: 10.1159/000487576]

40 Hou YJ, Dong LW, Tan YX, Yang GZ, Pan YF, Li Z, Tang L, Wang M, Wang Q, Wang HY. Inhibition of active autophagy induces apoptosis and increases chemosensitivity in
41 Nitta T, Sato Y, Ren XS, Harada K, Sasaki M, Hirano S, Nakanuma Y. Autophagy may promote carcinoma cell invasion and correlate with poor prognosis in cholangiocarcinoma. *Int J Clin Exp Pathol* 2014; 7: 4913-4921 [PMID: 25197362]

42 Hu F, Guo XL, Zhang SS, Zhao QD, Li R, Xu Q, Wei LX. Suppression of p53 potentiates chemosensitivity in nutrient-deprived cholangiocarcinoma cells via inhibition of autophagy. *Oncol Lett* 2017; 14: 1959-1966 [PMID: 28789429 DOI: 10.3892/ol.2017.6449]

43 Dong LW, Hou YJ, Tan YX, Tang L, Pan YF, Wang M, Wang HY. Prognostic significance of Beclin 1 in intrahepatic cholangiocellular carcinoma. *Autophagy* 2011; 7: 1222-1229 [PMID: 21654208 DOI: 10.4161/auto.7.10.16610]

44 Wang TT, Cao QH, Chen MY, Xia Q, Fan XJ, Ma XK, Lin Q, Jia CC, Dong M, Ruan DY, Lin ZX, Wen JY, Wei L, Li X, Chen ZH, Wang L, Wu XY, Wan XB. Beclin 1 deficiency correlated with lymph node metastasis, predicts a distinct outcome in intrahepatic and extrahepatic cholangiocarcinoma. *PLoS One* 2013; 8: e80317 [PMID: 24303007 DOI: 10.1371/journal.pone.0080317]

45 Yothaisong S, Dokduang H, Techasen A, Namwat N, Yongvanit P, Bhudhisawasdi V, Puapairoj A, Riggins GJ, Loilome W. Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy. *Tumour Biol* 2013; 34: 3637-3648 [PMID: 23832540 DOI: 10.1007/s13277-013-0945-2]

46 Thongchot S, Ferraresi A, Vidoni C, Loilome W, Yongvanit P, Namwat N, Isidoro C. Resveratrol interrupts the pro-invasive communication between cancer associated fibroblasts and cholangiocarcinoma cells. *Cancer Lett* 2018; 430: 160-171 [PMID: 29802929 DOI: 10.1016/j.canlet.2018.05.031]

47 Huang WK, Yeh CN. The Emerging Role of MicroRNAs in Regulating the Drug Response of Cholangiocarcinoma. *Biomolecules* 2020; 10 [PMID: 33007962 DOI: 10.3390/biom10101396]

48 Ma J, Weng L, Wang Z, Jia Y, Liu B, Wu S, Cao Y, Sun X, Yin X, Shang M, Mao A. MiR-124 induces autophagy-related cell death in cholangiocarcinoma cells through direct targeting of the EZH2-STAT3 signaling axis. *Exp Cell Res* 2018; 366: 103-113 [PMID: 29530475 DOI: 10.1016/j.yexcr.2018.02.037]

49 Lv P, Luo YF, Zhou WY, Liu B, Zhou Z, Shi YZ, Huang R, Peng C, He ZL, Wang J, Zhang HH, Nie SD. miR-373 inhibits autophagy and further promotes apoptosis of cholangiocarcinoma cells by targeting ULK1. *Kaohsiung J Med Sci* 2020; 36: 429-440 [PMID: 32125086 DOI: 10.1002/kjm2.12191]
