Chapter 11
Cell Death and Autophagy in Liver Tumorigenesis and Liver Cancer

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Abbreviations

3-MA	3-methyladenine
ATD	Alpha1-antitrypsin deficiency
ATG	Autophagy-related gene
CBZ	Carbamazepine
CQ	Chloroquine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCQ	Hydroxychloroquine
HCV	Hepatitis C virus
ICC	Intrahepatic cholangiocarcinoma
MDBs	Mallory-denk bodies
NAFLD	Nonalcoholic fatty liver disease

In recent years, autophagy has been a research focus. Cellular autophagy is particularly well studied in liver cancer, among the different tumor types. Confusingly, autophagy shows complex and dual roles in both suppressing and promoting tumors in liver cancer.

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11.1 Autophagy in Tumor Suppression

Autophagy was proposed to be a tumor suppressor due to the direct evidence, showing that liver tumors were developed in autophagy-related gene (ATG)-deficient mouse models. Currently, some critical autophagy genes, including beclin1, ATG5 and ATG7, are proven tumor suppressor genes. In an ATG5 knockout mouse model, multiple benign tumors were developed only in the liver, but not in other tissues, indicating that continuous autophagy is important for the suppression of tumorigenesis. Moreover, swollen mitochondria, oxidative stress, and genomic damage responses were detected in the hepatic tumor cells. Similar results were also observed in liver-specific ATG7-deficient mice (Takamura et al. 2011). Beclin1 is a mammalian autophagy gene and a haploinsufficient tumor suppressor and negatively regulates tumorigenesis (Liang et al. 1999). Beclin1$^{-/-}$ mutant mice not only died early in embryogenesis but also suffered from a high incidence of spontaneous tumors (Qu et al. 2003; Yue et al. 2003). Heterozygous disruption of beclin1 could increase the frequency of spontaneous malignancies and accelerate the development of hepatitis B virus (HBV)-induced premalignant lesions. In addition, beclin1 heterozygous disruption also leads to an increase in cellular proliferation and reduces autophagy in vivo (Qu et al. 2003). Recently, studies revealed that the expression of beclin1 and bax in hepatocellular carcinoma (HCC) tissues might provide a synergistic effect that inhibits tumor proliferation, infiltration, metastasis, and angiogenesis (Qiu et al. 2014). All of these data from animal models provide clear evidence that autophagy is an important suppressor mechanism to prevent tumorigenesis. p62 is a selective autophagy substrate. Autophagy defects could result in sustained expression of p62 and promote tumor progression (Mathew et al. 2009). In human cancers, p62 accumulation has been detected (Inami et al. 2011), it is involved in heterozygous mutations of the beclin1 gene (Komatsu et al. 2007; Mathew et al. 2009; Qu et al. 2003; Yue et al. 2003). In a mouse model, liver-specific ATG7 knockout mice developed hepatocellular adenoma accompanied by excessive p62 accumulation and subsequent Nrf2 activation, and persistent Nrf2 activation contributes to the HCC formation (Inami et al. 2011). After a concomitant knockout of the p62 gene, tumor size in this mouse model significantly decreased (Takamura et al. 2011). Furthermore, the tumor suppressor gene p53 plays a dual role in cellular autophagy. In the cytoplasm, autophagy is suppressed by p53, and p53 degradation is also required for the induction of autophagy (Levine and Abrams 2008). Additional evidence indicates that p62 is increased in HCC tissues compared with non-tumorous liver tissues, suggesting that human HCC is autophagy defective (Bao et al. 2014). The suppression of liver cancer is also reflected in another aspect: the autophagy level is negatively correlated with the degree of HCC malignancy. Under starvation conditions, decreased basal expression of autophagic genes and their corresponding autophagic activity in HCC cell lines, and autophagy defects are correlated well with the highly malignant phenotype (Ding et al. 2008). These results suggest that autophagy defects in combination with altered apoptotic activity might facilitate tumor progression and poor prognosis because autophagy might interact

with apoptosis in the regulation of HCC (Shi et al. 2009). Due to its suppression effect on liver tumor, autophagy might be of therapeutic value. Tissue injury, inflammation, and genomic instability are risk factors for carcinogenesis. In precancerous cells, these risk factors could be inhibited by autophagy, and autophagy induction may be beneficial for the prevention of liver cancer. For example, in patients with α-1 antitrypsin deficiency, inflammation and carcinogenesis could be inhibited by the upregulation of basal autophagy level in liver cells (Perlmutter 2009). It has been demonstrated that carbamazepine (CBZ) could increase the autophagy level, reduce the α-1 antitrypsin load, and lower the risks of liver fibrosis and cancer in an α-1 antitrypsin deficiency (ATD) mouse model (Hidvegi et al. 2010), indicating that increased autophagy might provide effective and preventive effects against liver cancer.

11.2 Autophagy in Cancer Promotion

Growing evidences indicate that autophagy is needed by tumor cells under disadvantageous conditions, including starvation, deficiency of growth factor, hypoxia, injury, and drug medication. Generally, up-regulation of basal autophagy is required for the survival of tumor cells, implying that cancer cells use the catabolic function of autophagy to tolerate stress. The autophagosome is common in ischemic areas, and the autophagy inhibition induced by decreased levels of beclin1 causes cellular death (Degenhardt et al. 2006). In RAS-driven tumors, the basal autophagy level is increased. Moreover, RAS-expressing ATG5$^{-/-}$ or ATG7$^{-/-}$ cells displayed reduced tumor growth in vivo (Guo et al. 2011).

Recently, increasing evidences showed that HCC metastasis could be suppressed by autophagy, and the intrinsic mechanism underlying metastasis might involve facilitating anoikis resistance and lung colonization (Peng et al. 2013b, c). Moreover, studies demonstrated that hypoxia-induced autophagy contributed to HCC chemoresistance. In vitro, by enhancing cellular survival and decreasing the apoptotic potential, autophagy may contribute to the resistance of HCC cells to chemotherapeutic agents under hypoxia; thus, the cellular survivability is changed by autophagy suppression (Song et al. 2009). Because autophagy is essential for tumor survival pathways, autophagy modulation is a novel approach for enhancing the efficacy of existing cancer therapy. Current (angiogenesis inhibitors and growth factor receptors) and conventional cancer treatments (radiotherapy and chemotherapy) could lead to metabolic stress and induce autophagy. To improve the clinical efficacy, autophagy inhibitors could be used to eliminate the protective effect of autophagy in combination therapy (Wu et al. 2012). Antimalarial drugs (chloroquine and hydroxychloroquine) have been evaluated and shown promising results in preclinical and clinical trials, and research on specific small molecule autophagy inhibitors is currently in progress (details as follows).

Autophagy could exert a strong influence on carcinogenesis and tumor progression by altering the risk factors of liver cancer, including HBV, hepatitis C virus

(HCV), alcohol intake, and others. HBV infection is the most important leading cause of liver cancer, and it induces autophagy by enhancing viral DNA replication with only a slight effect on viral RNA transcription (Sir et al. 2010). During a natural HBV infection, autophagy facilitates HBV replication, which has primarily been demonstrated in cell cultures and in a mouse model (Li et al. 2011; Tian et al. 2011). HCV is also correlated with liver cancer, and studies have shown that autophagy contributes to the self-replication of HCV particles. HCV RNA replication could promote the expression of UPR and CHOP, thus leading to the accumulation of autophagosomes and activation of autophagy (Ait-Goughoulte et al. 2008; Sir et al. 2008). Some ATGs (BECN1, ATG4B, ATG5, and ATG12) are involved in the translation of viral mRNA and initiation of replication (Dreux et al. 2009). HCV RNA replication may block the maturation of the autophagosome and autolysosome (Sir et al. 2008). Furthermore, HCV seems to avoid recognition by the autophagic machinery (Alavian et al. 2011; Rautou et al. 2010). Chronic alcohol consumption has been widely recognized as one of the most common causes, and it may lead to several types of liver status: fatty liver, hepatic fibrosis, cirrhosis, and alcoholic hepatitis. The mechanism underlying the liver injury may include the induction of oxidative stress. Hepatic steatosis and oxidative stress are regulated by autophagy in liver cells, indicating the importance of autophagy in ethanol-induced liver diseases (Czaja 2011; Wang et al. 2010). Needless to say, the exact role of autophagy in alcohol-related liver cancer remains to be further studied. Nonalcoholic fatty liver disease (NAFLD) is a common chronic liver disease. The loss of lipid droplets is promoted by autophagy and is called fat endocytosis (Singh et al. 2009). In hepatic stellate cells, blocking autophagy could result in the accumulation of lipid droplets and attenuation of liver fibrosis (Hernandez-Gea et al. 2012), implying that autophagy may prevent the formation of NAFLD-related tumors.

It should be noted that the majority of current research is focused on HCC, but autophagy also plays a similar role in two other types of liver cancer, including intrahepatic cholangiocarcinoma (ICC) and hepatoblastoma. In nutrient starvation and xenograft cholangiocarcinoma cells, autophagy is activated (Hou et al. 2011). Inhibiting autophagy (by an autophagy inhibitor or beclin1 siRNA) could induce apoptosis during starvation and increase the sensitivity of cholangiocarcinoma cells to chemotherapy (Hou et al. 2011). Similarly, blocking autophagy by regulating autophagic genes could suppress the formation of hepatoblastoma. However, the exact mechanism of autophagy in ICC and hepatoblastoma requires in-depth studies.

11.3 Autophagy as a New Therapeutic Target

Due to its critical role in liver cancer, autophagy has been considered a new therapeutic target, but there is currently no consensus about autophagy therapy in clinics. Theoretically, autophagy inhibitors could eliminate or weaken the protective roles of chemotherapy induced by autophagy, which could enhance the tumor-killing

effect and promote the therapeutic effect. In addition, autophagy inducers could lead to cellular autophagic death when combined with chemotherapy. By activating the PI3K-Akt and Akt signaling pathways, sorafenib or bortezomib treatment could activate autophagy, and combining these treatments with chloroquine (CQ) or 3-methyladenine (3-MA) could improve their cytotoxicity (Hui et al. 2012; Shimizu et al. 2012; Yu et al. 2013). Cisplatin and oxaliplatin share similar effects (Ding et al. 2011; Xu et al. 2012). However, the molecule machinery of certain drugs is not similar to those above. Anticancer drugs (nilotinib, cannabinoids tetrahydrocannabinol, JWH-015, and SAHA) can upregulate autophagy by activating the AMPK signaling pathway, but their treatment efficacy was decreased when they were combined with inhibitors. Autophagy may protect cancer cells, but the induced autophagy mediated by the AMPK signaling pathway seems to induce cytotoxicity. The above results indicate the complexity of autophagy treatments in liver cancer.

11.4 Autophagy Inducers and Inhibitors

Owing to the multi-faceted role of autophagy, inhibitor applications depend on the specific circumstances (Amaravadi et al. 2011, 2007). Autophagy is considered a cellular death mechanism that promotes apoptosis or autophagic cell death, leading to better treatment (Edinger and Thompson 2004; Eisenberg-Lerner et al. 2009; Shen and Codogno 2011). In apoptosis-deficient tumor cells, induced autophagy activity and increased autophagic cell death are effective ways to facilitate cellular death (Maycotte and Thorburn 2011). The autophagy inducers and inhibitors that have been developed using in vitro or in vivo systems and have been used in clinical trials are summarized in Table 11.1.

Autophagy inducers: important cellular autophagy inducers for liver cancer (based on the concept that autophagy could improve the treatment effect by enhancing cellular death) include the following: (1) rapamycin and its analogs: in HCC, the PI3K/Akt/mTOR pathway is the key signaling cascade because it modulates cellular growth, proliferation, angiogenesis, and apoptosis (Sabatini 2006), and this pathway is activated in 15–41% of HCC tumors. mTOR inhibitors have anti-HCC activity (Sieghart et al. 2007). Rapamycin (sirolimus), an mTOR inhibitor, is widely used as an autophagy inducer, and it has been applied for its anti-proliferation and anti-angiogenesis effects. In clinical research, rapamycin and its analogs (everolimus) have proven anti-tumor activity (Huynh et al. 2009). (2) Tyrosine kinase inhibitors: tyrosine kinase plays an important role in tumor progression, and it has been utilized in cancer treatment. In combination with the HDAC inhibitor SAHA, sorafenib can enhance liver cancer death by inducing autophagy (Martin et al. 2009; Park et al. 2008). However, adriamycin (DOX)-induced autophagic cell death can be inhibited by sorafenib, thus promoting the cell cycle progress, improving the survival rate, and decreasing cancer autophagy (Manov et al. 2011). Therefore, the potent antagonistic effect of sorafenib and adriamycin treatment needs to be further considered. (3) Others: a new naphthalimide polyamine conjugate

Table 11.1 Autophagy inducers and inhibitors used in clinical trials and developed in in vivo and in vitro systems

	Drug	Experimental system
Autophagy inducer	Rapamycin	C57BL/6 mice, Hep G2; mice
	Everolimus	Xenograft mouse model
	Everolimus + BEZ235	Hepatocyte cell line, mice, Stage I clinical trial
	Bortezomib	Huh7 cells/FVB and transgenic mice
	SAHA/OSU-HDAC42	Hep 3B, Hep G2, Huh7
	Panobinostat	Hep 3B, Hep G2, Huh7, human, xenograft mouse model
	Sorafenib	Hep 3B, Hep G2, Huh7
	Nilotinib	Hepatocyte cell line, mice
	NPC-16	Hep G2, Bel-7402
	Berberine	MHCC97-L, HepG2
	MLN4924	Huh7, Hep G2, xenograft mouse model
	Δ9-THC/JWH-015	Hep G2, Huh7, xenograft mouse model
	THC	Hepatocyte cell line, mice
	Fangchinoline	Hep G2, PLC/PRF/5
	SB203580	Hep G2, Hep3B, PLC/PRF/5, Huh7
	Melatonin	H22, H22 mouse models
	Cisplatin	Hepatocyte cell line, mice
	Oxaliplatin	Hepatocyte cell line, mice
	miR-100	Hepatocyte cell line, mice
Autophagy inhibitor	3-MA	H22, Hep G2; xenograft mouse model, PLC/PRF/5; SMMC7721
	Wortmannin	HCCC9810
	Bafilomycin A1	Hep 3B, Hep G2, Huh7; HA22T/VGH
	CQ/HCQ	HA22T/VGH; Mahlavu
	siRNA (siATG5, siBeclin1, siATG7, shBeclin1, shATG5)	Hep G2, H22; PLC/PRF/5; HA22T/VGH; Huh7, HCCLM3, MHCC97H, SMMC7721
	MicroRNAs (mir-375, miR-101, miR-199a-5p)	Huh7 and Hep3B, xenograft mice

can induce autophagy and apoptosis, and the involvement of the mTOR pathway in the autophagy mediated by NPC-16 has been confirmed (Xie et al. 2011). Berberine, which inhibits the mTOR pathway by up-regulating the AMPK signaling pathway, can also induce autophagic cellular death in liver cancer (Wang et al. 2010a; Yu et al. 2014). The cannabinoid Δ9-THC and its receptor agonist (JWH-015) could inhibit autophagy induction and decrease the growth of subcutaneous xenografts through the Akt-mTORC1 axis and stimulation of the AMPK pathway. Evidence demonstrated that Δ9-THC and the agonist JWH-015 led to the death of liver cancer. As a new type of antitumor agent, fangchinoline can induce autophagic death in liver cancer through the p53/sestrin2/AMPK pathway (Wang et al. 2011).

Furthermore, MLN4924 (a small molecule inhibitor of NEDD8-activating enzyme) can suppress the growth of liver cancer by inducing autophagy in vivo and in vitro. The intrinsic mechanism might be the suppression of mTOR activity by Deptor (mTOR-binding protein) (Luo et al. 2012).

Autophagyinhibitors: autophagy is an essential survival mechanism, through which cancer cells can survive under various stress conditions, including drug treatment. Therefore, inhibitors can increase the treatment effect by abolishing the protective effect of autophagy, increasing cytotoxicity, and limiting treatment resistance. (1) CQ and hydroxychloroquine (HCQ): CQ and HCQ are widely used as autophagy inhibitors. Many reports have demonstrated that drug treatments, including CQ and HCQ treatments, have a sensitizing effect on cell apoptosis both in vivo and in vitro. For example, CQ-induced autophagy increases the cellular death caused by oxaliplatin, improving the chemosensitivity in liver cancer, and a similar result was shown by ding et al. (Ding et al. 2011). In HCC cell lines and a nude mouse model, the sorafenib group combined with CQ significantly suppressed the tumor growth compared with that for sorafenib alone (Shi et al. 2011; Shimizu et al. 2012). Currently, 25 autophagy-related clinical trials are in progress that use CQ/HCQ alone or in combination with other drugs to treat tumors (http://www.clinicaltrials.gov). However, no related clinical trials are being performed for liver cancer. (2) siRNA and shRNA: due to the high specificity and selectiveness of siRNA or shRNA, side effects could be avoided to a large extent by silencing certain autophagy genes. Many reports have reported cancer treatments that knock out specific ATG genes, but there are no clinical trials targeting liver cancer. Chen demonstrated that suppressed autophagy, which was mediated by knocking down beclin1, promoted increased cellular death in liver cancer (Chen et al. 2011). Beclin1 silencing also facilitates the H22 cellular death induced by melatonin and improves the antitumor effects of melatonin (Liu et al. 2012). Overall, silencing-specific ATG genes to inhibit autophagy could improve the clinical effect of chemotherapy. However, it is still unclear whether the siRNA-induced treatment effect would occur in liver cancer. Via the construction of viral vectors carrying shRNA or miRNA targeting critical autophagy genes, autophagy inhibition in vivo might be a new field in the future. An autophagy-related and specific shRNA designed by Peng et al. was demonstrated to be of great value in suppressing autophagy and lung metastasis both in vivo and in vitro (Peng et al. 2013a, c). (3) microRNAs (miRNAs) can regulate autophagy through multiple genes and pathways (Kim and Kim 2014). miRNAs could regulate autophagy in liver cancer by acting on ATG genes. Many miRNAs (such as miR-101, miR-30a, miR-34a, miR-204, and miR-375) could serve as inhibitors. In liver cancer, hypoxia-induced autophagy could be suppressed effectively by the direct actions of miR-375 on ATG7 (Kim and Kim 2014). Transfection with a miR-375 mimic attenuated the protective effect of autophagy and impaired the vitality of tumor cells (Pastore et al. 2013). By down regulating STMN, RAB5A, and ATG4D, miR-101 could suppress HCC autophagy. When combined with adriamycin and fluorouracil, miR-101 could increase their chemosensitivity. In liver cancer patients treated with cisplatin, the miR-199a-5p level is decreased significantly; this miRNA acts on ATG7 and abolishes the drug resistance induced by autophagy.

Autophagy inhibition by miRNA is becoming a new focus. However, some miRNAs will induce autophagy. miR-100 facilitates HCC autophagy by preventing mTOR and IGF-1R expression. Overexpression of miR-100 can lead to cellular death, and this effect can be inhibited by ATG7 silencing and CQ, providing a potent target for HCC treatment. (4) Others: 20(S)-Ginsenoside Rg3 is a new type of autophagy inhibitor that can improve the adriamycin treatment effect (Kim et al. 2014). Adriamycin-induced autophagy has a protective effect in liver cancer. Ginsenoside Rg3 inhibits late-period autophagy. Combined with doxorubicin, Rg3 is able to kill HCC cells, which exhibit good tolerance to Rg3, and able to inhibit liver cancer xenografts in mice. The combination of inhibitors and traditional chemotherapy drugs is becoming an effective strategy in liver cancer treatment.

11.5 Conclusion

In liver cancer, autophagy has a Janus-face in that being a tumor survival mechanism, it can also lead to autophagic cell death. Accordingly, taking into account the essential role of autophagy in liver cancer and the existence of therapies targeting this process, may improve clinical efficacy of liver cancer. Exploring the potential clinical value of autophagy in liver cancer may lead to a new avenue of cancer therapeutics.

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