Review

Multiple Roles of Autophagy in the Sorafenib Resistance of Hepatocellular Carcinoma

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Key Words

Hepatocellular carcinoma • Sorafenib • Autophagy • Drug resistance

Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide, and prognosis remains unsatisfactory since the disease is often diagnosed at the advanced stages. Currently, the multikinase inhibitor sorafenib is the only drug approved for the treatment of advanced HCC. However, primary or acquired resistance to sorafenib develops, generating a roadblock in HCC therapy. Autophagy is an intracellular lysosomal pathway involved in protein and organelle degradation, with an astonishing number of connections to human disease and physiology. Current understanding of the role of autophagy in the progression of cancer and the response to cancer therapy remains controversial. Sorafenib is able to induce autophagy in HCC, but the effect of autophagy is indistinct. Some studies established that sorafenib-induced autophagy serves as a pro-survival response. However, other studies found that sorafenib-induced autophagy improves the lethality of sorafenib against HCC cells. The mechanisms underlying autophagy and sorafenib resistance remain elusive. The purpose of this review is to summarize the progress of research focused on autophagy and sorafenib resistance and to update current knowledge of how cellular autophagy impacts sorafenib sensitivity in HCC treatment.

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Introduction

Liver cancer is a major health problem and a primary cause of cancer-related death worldwide, only second to lung cancer. Hepatocellular carcinoma (HCC), the most common type of liver cancer, is the fifth most commonly diagnosed cancer and the second most common cause of cancer deaths worldwide [1]. HCC commonly develops in patients with underlying chronic liver inflammation related to viral infection, alcohol intake, or metabolic syndrome [2, 3]. The recommended treatment of HCC patients depends on general status

of the patient, assessment of current liver function, tumor size, vascular invasion, and the presence of extrahepatic metastasis. Despite remarkable progress in the prevention, diagnosis, and treatment of HCC throughout the last several decades, there is still no sufficiently effective treatment due to late-stage diagnosis and inadequate clinical strategies for inhibiting metastasis [4]. Potentially curative treatments such as surgical resection and liver transplantation may be available for patients diagnosed at early stage [5]. For patients with advanced HCC, only palliative treatments are available, and sorafenib is the only choice of systemic therapy [6, 7].

Sorafenib (BAY 43-9006, Nexavar®), an oral multikinase inhibitor, remains the only FDA-approved systemic drug for patients with advanced HCC [8-10]. Sorafenib was approved for the treatment of patients with advanced HCC on the basis of two international randomized, controlled trials (RCTs), the SHARP (Sorafenib HCC Assessment Randomized Protocol) and the Asia-Pacific trials [11, 12]. The SHARP trial, conducted in western countries, was a multicenter, phase III, double-blind study, in which 602 HCC patients who had not received any previous systemic treatment were assigned to receive sorafenib 400 mg bis in die (bid) or placebo. Sorafenib prolonged the median overall survival (OS) to 10.7 months versus 7.9 months in the placebo group [11]. In the Asia-Pacific trials, 226 patients with HCC were randomized to receive sorafenib 400 mg bid or placebo. In the sorafenib group, the median OS was 6.5 months, compared with 4.2 months in the placebo group [12]. It is worth noting that, patients in the SHARP trial were mostly of Caucasian ethnicity and had a high prevalence of HCV infection; conversely, patients in the Asia-Pacific trial were more largely infected by HBV and presented a more advanced stage of HCC and a worse liver function. These differences can explain the difference in OS observed between the two trials.

Sorafenib suppresses tumor angiogenesis and proliferation by inhibiting serine/threonine kinases and receptor tyrosine kinases [13]. Sorafenib displays a remarkable inhibition of the RAF/MEK/ERK and p38/MAPK signaling pathways [14, 15] and also suppresses other receptor tyrosine kinases such as VEGFR (vascular endothelial growth factor receptor), PDGFR (platelet-derived growth factor receptor), FLT-3 (FMS-like tyrosine kinase 3) and FGFR-1 (fibroblast growth factor receptor-1) [16, 17].

Nevertheless, a considerable number of HCC patients are refractory to sorafenib treatment. Sorafenib is beneficial in only approximately 30% of patients, and acquired resistance often develops within 6 months [11, 12], suggesting the existence of primary and acquired sorafenib resistance in HCC cells. Sorafenib resistance is mediated through multiple factors that have not been thoroughly investigated. It is thought to be largely due to upregulation of survival pathways that may neutralize the death signals induced by sorafenib. Therefore, a better knowledge of these cellular pathways is required to overcome tumor resistance and consequently improve the effects of sorafenib therapy [18-20].

Autophagy and cancer

Autophagy is an evolutionarily conserved process in which damaged or useless organelles, proteins or other cytoplasmic components are delivered to lysosomal system for degradation [21-23]. It has been characterized as an adaptive catabolic process that is essential for maintaining cellular homeostasis [24, 25]. Various stimuli, such as nutrient deprivation, growth factor withdrawal, hypoxia or drug treatment, have the ability to induce autophagy [26]. Autophagic process involves a highly conserved group of genes which were discovered in yeasts and named as autophagy-related genes (Atgs) (Fig. 1) [27-29]. Autophagy is initiated by the ULK1-Atg13-FIP200 complex [30]. Next, Beclin1-Vps34-Atg14 complex contributes to membrane nucleation and elongation [31, 32]. Besides, two ubiquitin-like systems, Atg5-Atg12-Atg16 and LC3, are both required for elongation of the autophagosome membrane. Closure of the autophagosome is dependent on the activity of the LC3-conjugation system [33]. In most cases, the formed autophagosomes merge with endocytic pathway and then fuse with lysosomal compartment. These steps are collectively

Fig. 1. Schematic model of autophagy flux. Autophagy is initiated by the nucleation of an isolation membrane or phagophore (Initiation). The process is initiated by the ULK1-Atg13-FIP200 complex. This membrane then elongates to engulf cytoplasmic components (Elongation). Beclin 1: Vps34: Atg14 complex contributes to the formation of an isolation membrane and elongation. Elongation of the phagophore membrane is dependent on the Atg5-Atg12-Atg16 and LC3 conjugation systems. LC3-II localizes to the elongated isolation membrane at the latter step of autophagosome formation, while the Atg5-Atg12-Atg16 complex dissociates from it. Closure of the autophagosome is dependent on LC3- II. Finally, the isolation membrane is enclosed to form an autophagosome. After autophagosome formation, the lysosome fuses with the autophagosome to form an autolysosome (Autophagosome-lysosome fusion). Intra-autophagosome contents are degraded by lysosomal hydrolases (Degradation). The Beclin 1:hVps34:UVRAG complex positively regulates the fusion and degradation. When associated with Rubicon, it inhibits autophagic degradation.

termed maturation. The Beclin1-Vps34-UVRAG complex positively regulates the maturation of autophagosomes and endocytic trafficking. When associated with Rubicon, the complex blocks autophagosome maturation [34, 35]. Different signaling pathways participate in autophagy regulation. The most important pathways are related to mTORC1 (mammalian target of rapamycin complex 1) and AMPK (5'AMP-activated protein kinase). mTORC1 abolishes autophagy in the presence of abundant nutrients or growth factors through inhibition of ULK1, while AMPK induces autophagy when cellular energy is insufficient [36, 37].

Autophagy acts as a double-edged sword in cancer cells [38, 39]. Basal autophagy acts as a tumor suppressor by maintaining genomic stability in normal cells. Once cancer has been established, activated autophagy will contribute to cancer cell survival under stress and in turn promote cancer development [40-43]. Studies have indicated that, in different cancers, autophagy appears to serve as either a pro-survival or pro-death response. Autophagy is also considered as an important mechanisms of drug resistance by supporting the survival of tumor cells under metabolic and therapeutic stress. During cancer treatment, including radiation, chemotherapy and target therapy, autophagy is usually activated [44]. Thus, further studies of autophagy and sorafenib is highly valuable in cancer treatment, and resistance to sorafenib must be resolved to improve sorafenib efficiency and HCC treatment.

Table 1. Sorafenib-induced autophagy in HCC

Cell line	Related signaling pathway	Effects on autophagy and cancer	References
MHCC97-L, PLC/PRF/5, HepG2	IRE1 signaling pathway of ER stress	Inducing autophagy and alleviating the ER stress-induced cell death.	[45]
HepG2, Huh7	Activating Akt via the feedback loop of mTOR	Inducing autophagy and resistance to sorafenib	[46]
Huh7, HLF, PLC/PRF/5	Inhibiting mTORC1	Inducing autophagy and resistance to sorafenib	[47]
PLC5, HepG2	Releasing Beclin1 from binding with Mcl-1	Inducing autophagic cell death	[48]
Sk-Hep1, Hep3B	Activating AMPK and consequently autophagy in Huh7, but not in Hep3B cells.	Different autophagy responsiveness to sorafenib might be linked to generation of sorafenib resistance.	[49]

Sorafenib induces autophagy in HCC

Sorafenib is a multi-kinase inhibitor that targets multiple genes that modulate the cellular survival, proliferation and metastasis of cancer. Studies have shown that sorafenib treatment enhances autophagy in HCC cells (Table 1). Shi et al. found that sorafenib induced both apoptosis and autophagy in human HCC cells through upregulation of IRE1 signals that involves endoplasmic reticulum (ER) stress [45]. Sorafenib can also induce autophagy through activating Akt pathway or inhibiting mTORC1 [46, 47]. Moreover, according to the study by Tai et al., sorafenib activated autophagy through releasing Beclin1 from binding with Mcl-1 [48].

It is obvious that autophagy is responsible for orchestrating adaptive responses to sorafenib in HCC. However, the effect of autophagy is indistinct. Some studies have established that sorafenib induces the activation of protective autophagic responses in HCC cells. Inhibition of autophagy using either pharmacological inhibitors (chloroquine, 3-MA or bafilomycin A1) or essential autophagy gene (Beclin1 or Atg5) knockdown enhances the cytotoxicity of sorafenib against HCC cells, indicating that autophagy induced by sorafenib acts as a protective pathway [45-47, 50, 51]. Patients with higher Atg7 expression and autophagy activity have a poorer prognosis for sorafenib treatment [52]. However, not all sorafenib-induced autophagy serves as a pro-survival response, and other studies have observed opposite results in HCC. Studies have reported that sorafenib-induced autophagy improves the lethality of sorafenib against HCC cells [48]. Furthermore, various HCC cell lines exhibit different sensitivity to sorafenib might due to different autophagy responsiveness to sorafenib [49].

Animal models are viewed as crucial tools in the study of cancer. To clarify the pathogenesis of HCC and investigate the effects of potential therapies, a number of animal models have been developed. Considered as an uncontrollable and unpredicted system, with an extremely low incidence rate, spontaneous HCC models are scarcely used nowadays. Subcutaneous xenograft models are widely used in the past few decades. Development of transgenic and gene targeting technologies facilitated the generation of genetically engineered mouse models (GEM), which have greatly facilitated studies of gene function in HCC development. The most common ways to generate mouse models of HCC are activating oncogenes (such as Ras, HBx, c-Met, c-Myc or AKT) or inactivating tumor-suppressor genes (p53, Rb), or both *in vivo* through the use of transgenic and gene-targeting approaches [53-58]. Moreover, chemically induced hepatocarcinogenesis in animals share many of the morphological, histogenic, and biochemical features of human HCC, which begin with an irreversible process characterized by structural DNA changes [59]. Diethylnitrosamine (DEN), carbon tetrachloride (CCl₄), aflatoxine, dimethylnitrosamine (DMN) and thioacetamide are common hepatic carcinogens. Among them, DEN-induced HCC model is the most frequently used [60, 61]. A recent study have demonstrated that autophagy inhibits HCC progression and sorafenib resistance by promoting degradation of HIF1 α in the DEN-induced HCC mouse model [62].

The pathways involved in sorafenib-mediated autophagy regulation

There are many proteins and pathways that are involved in sorafenib-mediated autophagy regulation in HCC cells (Fig. 2). mTORC1 is a highly conserved serine/threonine kinase complex and a key negative regulator of autophagy [63, 64]. Some researchers have assessed the role of mTORC1 suppression in sorafenib-induced autophagy in HCC [47]. p70S6K and 4E-BP1 are proteins downstream of mTORC1 [65]. Furthermore, sorafenib is able to dephosphorylate p70S6K and 4E-BP1 in HCC cells and in HCC xenograft models, indicating an inhibition of mTORC1 activity [66]. The PI3K/Akt signaling pathway is a major upstream inducer of mTORC1. Studies have shown that activation of PI3K/Akt signaling pathway mediates acquired resistance to sorafenib in HCC cells [67]. Furthermore, another study demonstrated that inhibition of Akt reverses acquired resistance to sorafenib by switching protective autophagy to autophagic cell death in HCC [46].

AMPK is an important energy sensor in cells [68, 69]. AMPK can initiate autophagy through inactivation of mTORC1 or by phosphorylation of ULK1 [70, 71]. It is possible that sorafenib activates AMPK by inducing cellular ATP reduction and a corresponding increase in the AMP/ATP ratio [72, 73]. Curiously, there are differences in sorafenib-dependent modulation of AMPK in HCC cell lines, where AMPK and consequently autophagy were activated in Huh7, but not in Hep3B cells. Hep3B cells were significantly more resistant to sorafenib than Huh7 cells [49]. Therefore, different autophagy responsiveness to sorafenib might be linked to generation of sorafenib resistance. Nevertheless, the AMPK pathway may be not involved in sorafenib-induced autophagy, since LC3-II was still upregulated significantly when AMPK was inhibited in HCC cells cultured with sorafenib [73, 74].

Beclin1 is a very important protein in autophagy initiation [31, 75]. Beclin1 contains a BH3 domain which binds to multiple antiapoptotic proteins belonging to the Bcl-2 family, such as Bcl-2, Bcl-xL, or Mcl-1 [76]. This interaction represses Beclin1 activity and significantly reduces initiation of autophagy. When the BH3 domain is phosphorylated, there is decreased binding affinity between these two proteins, resulting in Beclin1 release and its subsequent activation [76, 77]. A study found that sorafenib reduces the phosphorylation of STAT3, which subsequently reduces the downstream gene Mcl-1 expression. As a result, the Mcl-1-Beclin1 interaction was disrupted, inducing the release of Beclin1 and the formation of new autophagosomes [48]. It demonstrated that sorafenib activates autophagy in HCC cells through the STAT3-Mcl-1-Beclin1 pathway [48].

Other genes promoting sorafenib resistance through regulating autophagy

A study explored the mechanism of PSMD10-induced autophagy in sorafenib resistance. Increased expression of PSMD10 and Atg7 is a marker of poor prognosis in HCC patients receiving sorafenib treatment. PSMD10 is physically associated with Atg7 in the cytoplasm. Further study found that PSMD10 translocates to the nucleus and binds cooperatively with nuclear HSF1 (heat shock transcription factor 1) onto the Atg7 promoter to initiate its transcription [52]. Therefore, PSMD10 promotes autophagic flux and sorafenib resistance in HCC treatment and inhibition of autophagy could effectively reverse sorafenib resistance enhanced by PSMD10 [52].

Another report demonstrated that ADRB2 promotes sorafenib resistance by inhibition of autophagic degradation of HIF1 α . ADRB2 signaling negatively regulates autophagy by disrupting the Beclin1-VPS34-Atg14 complex in an Akt-dependent manner. Autophagy inhibition by ADRB2 leads to stabilization of HIF1 α and the acquisition of resistance to sorafenib. Conversely, inhibition of ADRB2 leads to enhanced autophagy, HIF1 α destabilization, and an improved anti-tumor activity of sorafenib. These results uncover an important role of ADRB2 signaling in sorafenib resistance through inhibition of autophagy [62].

MicroRNAs involved in autophagy and sorafenib resistance

MicroRNAs (miRNAs) are single-strand, small noncoding (~20nt) RNA molecules that bind to the 3'-untranslated region (3'UTR) of target genes and regulate their expression

Fig. 2. Pathways involved in sorafenib-mediated autophagy in HCC. Sorafenib induces AMPK pathway through increasing the AMP/ATP ratio, then initiates autophagy through inhibiting mTORC1 or by activating ULK1. mTORC1 can be inhibited by sorafenib through the ERK pathway. However, mTORC1 can also be activated by sorafenib through activating Akt. Sorafenib stimulates ER stress, which induces autophagosome formation. It may also disrupt Beclin1-Mcl-1 complex through inhibiting STAT3, releasing Beclin1 to activating autophagy. Sorafenib promotes PSMD10 translocating to the nucleus and initiates Atg7 transcription and autophagy. miRNAs can also be involved in sorafenib-mediated autophagy regulation. Sorafenib increases expression of miR-21, which suppresses autophagy through downregulating PTEN expression and sequential Akt activation. →, positive regulation or activation; ⊥, negative regulation or blockade.

It may also disrupt Beclin1-Mcl-1 complex through inhibiting STAT3, releasing Beclin1 to activating autophagy. Sorafenib promotes PSMD10 translocating to the nucleus and initiates Atg7 transcription and autophagy. miRNAs can also be involved in sorafenib-mediated autophagy regulation. Sorafenib increases expression of miR-21, which suppresses autophagy through downregulating PTEN expression and sequential Akt activation. →, positive regulation or activation; ⊥, negative regulation or blockade.

at the post-transcriptional level [78]. MiRNAs participate in multiple biological processes, including autophagy [79-81]. Recent studies have shown that miR-423-5p is upregulated in serum of HCC patients treated with sorafenib and higher expression of secretory miR-423-5p is correlated with an improved prognosis. Further experiments demonstrated that miR-423-5p regulates cell cycle progression and enhances autophagy flux. These data suggest that miR423-5p can be used as a useful tool to predict response to sorafenib treatment among HCC patients [82]. Another study showed that exposure of HCC cells to sorafenib leads to increased expression of miR-21. MiR-21 suppresses autophagy through downregulating PTEN expression and sequential Akt activation. Transfection of miR-21 mimics results in restored sorafenib resistance by inhibiting autophagy in HCC cells. These results demonstrated that miR-21 mediates sorafenib resistance of HCC cells by inhibiting autophagy via the PTEN/Akt pathway [83]. They also suggest that miR-21 could serve as a therapeutic target for overcoming sorafenib resistance in the treatment of HCC.

In summary, miRNAs are able to mediate autophagy in response to sorafenib and change the outcome of sorafenib treatment. They might also be predictors of the response of HCC patients to sorafenib treatment in the future.

Effect of sorafenib combination with other drugs on autophagy in HCC

Combination therapy using different pharmaceuticals has been proven highly effective due to the ability to affect multiple cellular pathways involved in cancer [84, 85]. Many studies have analyzed changes in autophagy modulation and effects on cancer treated with different drugs combination with sorafenib (Table 2).

Chloroquine, a lysosomotropic agent that prevents endosomal acidification, has long been used in the treatment or prevention of malaria [90]. Chloroquine inhibits autophagy as it raises the lysosomal pH, which leads to inhibition of fusion of autophagosome with lysosome and lysosomal protein degradation [38, 91]. Combination of sorafenib with chloroquine significantly suppressed tumor growth compared with sorafenib alone [45, 47]. Vorinostat is the first histone deacetylases (HDAC) inhibitor to be approved for human clinical use [92]. Vorinostat can sensitize HCC cells to sorafenib treatment by regulating the acetylation level of Beclin1. Autophagy inhibition significantly enhances combination therapy of sorafenib with

Table 2. Combination of sorafenib with another drug modulating autophagy in HCC

Drug combined with sorafenib	mechanism of action	Global effects on cancer	Reference
Chloroquine	Inhibiting autophagy degradation	more cell death and tumor suppression	[45]
Chloroquine	inhibition of autophagy degradation	increased apoptosis and suppressed tumor growth	[47]
Metformin	Inducing more autophagy disrupting the mTORC1/mTORC2 feedback loop	impaired proliferation and tumor inhibition	[50]
Vorinostat	Inhibiting autophagy via upregulating the acetylation level of Beclin 1.	apoptosis and cell cycle alterations	[51]
Pemetrexed	Inducing more autophagy through inhibition of AKT, p70 S6K and/or mTOR	more tumor killing via autophagic cell death	[86]
Akt inhibitor GDC0068	Switching autophagy from a cytoprotective role to a death-promoting mechanism via inhibiting Akt	reversing sorafenib resistance	[46]
Arsenic trioxide	diminishing the increased activation of Akt by sorafenib	inhibit the proliferation and promote the apoptosis of HCC cells	[87]
PHY906	affect autophagy by increasing AMPK α -P and ULK1-S555-P	enhanced the antitumor activity of sorafenib by changing the tumor microenvironment.	[88]
Doxorubicin	MEK/ERK counteraction, stimulation of survival via Akt and dysregulation of cyclin D1	escape from doxorubicin-induced autophagic cell death and thus promote cancer cell survival.	[89]

vorinostat for HCC [51]. Metformin is the first-line medication for the treatment of type II diabetes [93]. As an activator of AMPK, metformin should potentially induce autophagy [94]. Metformin and sorafenib cooperated to promote apoptosis and autophagy in HCC cells. The metformin and sorafenib combination led to increased cell death and tumor inhibition of HCC, which was partially bridged by disrupting the mTORC1/mTORC2 feedback loop [50]. The antifolate drug pemetrexed (ALIMTA, Lilly) has been approved by the FDA for the treatment of nonsmall cell lung cancer (NSCLC) and has been shown to stimulate autophagy [95, 96]. Studies noted that pemetrexed interacts with sorafenib to kill a wide variety of tumor cell types in a greater than additive fashion via autophagic cell death [86].

Studies have provided evidences that sustained exposure to sorafenib induces the activation of Akt, which contributes to the resistance to sorafenib in HCC cells. Inhibition of Akt reversed sorafenib resistance by switching cytoprotective autophagy to a death-promoting mechanism in the sorafenib-resistant HCC cells. GDC0068, a novel ATP-competitive pan-Akt inhibitor, could synergize with sorafenib to suppress the growth of sorafenib-resistant HCC [46]. Arsenic trioxide (ATO) is a currently clinically used anticancer drug and displays its anticancer activity by inhibiting Akt activation. Consequently, ATO potentiates the anti-cancer activities of sorafenib against HCC by diminishing the increased activation of Akt by sorafenib [87]. Sorafenib has also been combined with PHY906, a four-herb Chinese medicine formula. PHY906 can sensitize nude mice bearing HepG2 xenografts to sorafenib. The mechanism might be related to autophagy and microenvironmental activation [88].

However, not all drugs combination with sorafenib can promote anti-tumor activity of sorafenib. Doxorubicin alone could arrest proliferation and induce autophagic cell death in Hep3B cells. When combination with sorafenib, however, sorafenib facilitated cell cycle progression, increased survival and reduced autophagic cell death. Therefore, sorafenib acts as an antagonist of doxorubicin [89].

These studies have provided evidence for clinical investigation of some drugs as potential agents that given in combination with sorafenib acts synergistically for HCC therapy.

Conclusion

Hepatocellular carcinoma, the most common primary liver tumor, is notoriously resistant to systemic therapies. Sorafenib is the only FDA-approved target drug for advanced HCC, but development of resistance alters the efficiency of sorafenib therapy. Autophagy plays a complex role in cancer occurrence and development and is important in cancer therapy. In this review, we summarize the how sorafenib alters autophagy in HCC cells, and the influence of autophagy in the generation of sorafenib resistance. Sorafenib induces autophagy in HCC, causing either cell death or survival. Additionally, there are diverse cellular pathways involving proteins and miRNAs that influence the final outcome of autophagy in response to sorafenib treatment. Further study is needed to elucidate the relationship between autophagy and sorafenib resistance. Firstly, we should figure out which pathways were involved in sorafenib-induced autophagy and why autophagy was activated to different degrees in different HCC patients. Next, we should clarify why autophagy play different roles in HCC, promoting cell death or survival, and find out the underlying determinants of these different effects. Clarifying these questions will help to increase drug effectiveness and provide more effective treatment options for HCC patients.

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Disclosure Statement

The authors have declared that no competing interest exists.

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