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The role of Autophagy in Hepatocellular Carcinoma

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Abstract

Autophagy is a cellular catabolic process in which cytoplasmic material is delivered to lysosomes for degradation. The autophagy process is regulated by highly conserved autophagy-related genes (ATGs) via different signalling pathways. Among the various biological functions of autophagy, the link between autophagy and cancer has been extensively studied, demonstrating its dual role, of tumor suppressor or promoter in cancer development. Hepatocellular carcinoma (HCC) is one of the most lethal cancers that affects most of the world's population and it is caused by different etiological factors: HBV and HCV viral infections, heavy alcohol consumption, NAFLD (non-alcoholic fatty liver disease), aflatoxin B1 contaminated food. In recent years, the involvement of autophagy in both prevention and promotion of liver cancer has been increasingly studied. Here, we summarize molecular mechanisms and physiological function of liver autophagy, its dual role and its therapeutic potential in HCC.

Keywords: Hepatocellular carcinoma, autophagy

1. Introduction

The word Autophagy derives from the Greek roots "auto"(self) and "phagy" (eating) and was described for the first time by Cristian De Duve in the 1963 (Ravikumar, Sarkar et al. 2010).

Autophagy is a cellular catabolic process in which cytoplasmic material is delivered to the lysosome for degradation.

Intracellular components must be recycled to maintain energy and to ensure quality control of proteins and organelles, thus allowing cells to control homeostasis (Klionsky and Emr 2000, Klionsky 2007). Several types of factors including low ATP levels, nutrient and growth factor deficiency, hypoxic conditions, endoplasmic reticulum (ER) stress, pathogen entry or anticancer drugs may further upregulated autophagy (Yang and Klionsky 2010). In light of the multiple ways by which autophagy participates in the control of cell homeostasis, it is no surprise to observe alterations of this process concerning the pathogenesis of different diseases, such as neural degeneration, inflammatory bowel disease, aging and cancer (Chen and Karantza 2011). The latter scenario has been extensively studied (Kondo, Kanzawa et al. 2005, Jin and White 2007, Mathew, Karantza-Wadsworth et al. 2007, Eskelinen 2011, Rubinsztein, Codogno et al. 2012, Wu, Coffelt et al. 2012), demonstrating the dual role of autophagy in cancer development. Autophagy can act as tumour suppressor by i) inhibition of inflammation, ii) prevention of p62/SQSTM1(sequestosome 1) accumulation and iii) promotion of genomic stability. On the other hand, autophagy can promote cancer cell survival, protecting cancer cells from different cellular stress responses, including starvation, oxidative stress and DNA damage(White and DiPaola 2009).

Hepatocellular carcinoma (HCC) is one of the most common primary liver malignancy and a leading cause of cancer-related death worldwide (Liu, Liao et al. 2017). Currently, surgical resection is recommended for very early stage and early stage of HCC, following chemo/radiotherapy. However, these approaches are limited and not curative. HCC re-

currence and metastasis are observed after surgery (He, Lei et al. 2012, Ma, Wang et al. 2015, Sheng, Qin et al. 2018). Therefore, the understanding of precise contributions of deregulated molecular and cellular alterations it is essential to delineate the mechanism of tumorigenesis.

The most prominent aetiological factors associated with HCC development are: i)chronic hepatitis B (HBV) and C (HCV) viral infections; ii) chronic alcohol consumption and iii) aflatoxin B1 contaminated food (Liu, Liao et al. 2017). Besides, non-alcoholic and alcoholic fatty liver disease both contribute to the development of HCC.

Based on different studies autophagy appears to have a significant role in hepatocellular carcinogenesis. This suggestion is supported by the observations that autophagy is either involved in HCC promotion or prevention. This means that the overall functional effect on cancer pathology is likely to be dependent on multiple factors that may also influence dynamically the proposed autophagy-cancer model (Shintani and Klionsky 2004, White and DiPaola 2009, White 2015). Autophagy allows carcinoma cells to survive in the tumor microenvironment under stress condition including chemotherapies. On the other hand, it suppresses tumor initiation in healthy liver by ensuring the normal function of cells. Unfortunately, the exact mechanisms of autophagy regulation, in hetapocarcinogenesis, are not fully understood until now.

In this review, we present a brief overview of molecular mechanisms and physiological functions of autophagy. Moreover, we highlight recent data that describe the dual role of autophagy in HCC. Therapeutic approaches aimed at modulating autophagy are also discussed.

2. Mechanism and physiological role of autophagy

Autophagy process consists of three main steps: *initiation, elongation, maturation and fusion* of a double-membrane vesicle called *phagosome*. These steps are regulated by a series of highly conserved autophagy related genes (ATGs) via different signalling pathways (Yang and Klionsky 2009). The process starts with the formation of a double membrane vesicle known as *auto-phagosome* that engulfed cytoplasmic molecules. The membrane sources for auto-phagosome formation are ER, mitochondria and plasma membrane. The initiation of the auto-phagosome is under the control of two macromolecular complexes: mTOR-Atg13-ULK1 complex that initiates the generation of the two isolated membranes which extend to form phagophore, and PI3K complex (composed of Beclin1, Vps34, p150, Ambra1, UVRAG) that instead recruits the subsequent ATG proteins onto phagophore membrane(Liu, Liao et al. 2017).

The second step is characterized by the elongation of the auto-phagosome. This process involves two ubiquitin like conjugation systems, Atg5-Atg12 conjugation and LC3 phosphatidylethanolamine (PE) conjugation.

In the maturation step, auto-phagosomes that in mammalian are formed randomly in the cytoplasm, move bidirectionally along microtubules, preferentially towards the microtubule organization center (MTOC), where the lysosome are enriched. Auto-phagosome first fuse with endosomes and then with lysosome where the sequestered contents undergo degradation. The fusion machinery is recruited on the auto-phagosomes thanks to UVRAG and Beclin1 interacting proteins (Parzych and Klionsky 2014). In this way, the degradation products of cytoplasm portions will be cycled for energy generating and substrate supplying (Glick, Barth et al. 2010).

In the last years, considerable data have been accumulated about the physiological role of autophagy in the liver e.g. clearing misfolded proteins, regulation of nutrient and energy metabolism in hepatocytes, selective organelle degradation, and lipid and alcohol metabolism. Autophagy alterations may likely have a significant functional impact on all these processes.

2.1. Autophagy in clearing misfolded proteins

Autophagy together with the ubiquitin proteasome systems is involved in the control of intracellular protein homeostasis (Marfany, Farràs et al. 2008, Knævelsrud and Simonsen 2010). Piece of evidence from *Atg7* gene deficient mice showed accumulation of polyubiquitinated proteins and deformed mitochondria, as well as an increasing number of peroxisomes and lipid droplets in hepatocytes (Komatsu,

Waguri et al. 2005). Furthermore, *Atg7* deficient mice developed hepatomegaly and hepatic failure, thus suggesting the important role of autophagy in liver metabolism (Komatsu, Waguri et al. 2005).
Similar effects were also observed after the loss of *Vps34*, a gene that is essential in the autophagosome formation (Jaber, Dou et al. 2012).
Recent studies have provided evidence that autophagy may play a fundamental role in the degradation of alpha-1-antitrypsin (ATZ) that causes protein misfolding, pulmonary emphysema, chronic liver inflammation and HCC (Kamimoto, Shoji et al. 2006, Perlmutter 2006).
Until now, remains unclear how autophagy recognizes and removes the misfolded proteins, even though a recent study suggests the involvement of the UPR (unfolding protein response) pathway (Ding and Yin 2008).

2.2. Autophagy in organelle degradation

Autophagy is involved in organelle sequestration and turnover in hepatocytes. During autophagy process, the degradation rate of mitochondria, endoplasmic reticulum, membranes, ribosomes and Golgi apparatus is different from each other, thus indicating a specificity and selectivity of the autophagy process in various cellular constituents. Indeed, the two terms "mitophagy" and "ERphagy" were coined to imply the two selective processes in removing the mitochondria and ER. The exact molecular mechanism for mitophagy in liver cells remains to be further clarified, but it is clear that autophagy disruption causes mitochondrial dysfunction with a consequent increase in the reactive oxygen species (ROS) generation and DNA damage (Kim, Rodriguez-Enriquez et al. 2007).
The first evidence of ERphagy was established with the observation that extra smooth ER membranes could be degraded by autophagic vesicles selectively (Kanai, Watanabe et al. 1993).

2.3. Autophagy and nutrient stress

The most important and efficient inducer of autophagy is nutrient stress. In animal liver, starvation causes the largest proportion of protein loss (Addis, Poo et al. 1936), that determines a transient increase in the aminoacid levels in the liver tissue and blood after 24h in wild type mice but not in *Atg7* deficient mice. Aminoacids supplied during autophagy can be used for energy providing by means the tricarboxylic acid cycle (TCA) thus contributing to the metabolic requirements of cells. This data highlights the important role of autophagy in protein degradation in the liver. Similarly, the glucose level in the blood was stable after 24h of starvation in wild type mice, while *Atg7* deficient mice displayed hypoglycaemia (Ezaki, Matsumoto et al. 2011). Under starvation, free fatty acids in the liver can be esterified into triglycerides in lipid droplets which will be selectively degraded by autophagy to supply energy production through β-oxidation (Singh, Kaushik et al. 2009, Kaushik, Rodriguez-Navarro et al. 2011).
The suppression of autophagy in *Atg7* deficient mice shows accumulation of triglycerides and cholesterols in lipid droplets, thus indicating an important role in lipolysis blocking (Singh, Kaushik et al. 2009).

2.4. Autophagy and energy metabolism

It is well established that autophagy process is ATP dependent and depletion of ATP will impair autophagy. Under energy-low conditions including starvation, the energy sensor AMP-activated protein kinase (AMPK) acts as a metabolic checkpoint in inhibiting cellular growth and promoting autophagy (Hardie 2011).
AMPK can activate autophagy by at least two mechanisms: through the activation of ULK1, and through the inhibition of the suppressive effect of mTORC1 (Mihaylova and Shaw 2011).
Furthermore, it has been demonstrated that AICAR an analogue of AMP (adenosine monophosphate), suppressed autophagic sequestration of lactose dehydrogenase in hepatocytes, thus indicating the energy level is critical for autophagy regulation in the liver (Samari and Seglen 1998).

3. Implication of Autophagy in Hepatocarcinogenesis

The neoplastic evolution of HCC proceeds through a multi-step histological process that is less well defined with respect to other cancer types. Various risk factors are involved in the HCC onset including:

- chronic hepatitis B and C viral infections;
- heavy alcohol consumption;
- ingestion of aflatoxin B1;
- NAFLD (non-alcoholic fatty liver disease);
- diabetes;
- obesity;
- genetic disorders such as hemochromatosis.

These different HCC-inducing aetiologies provoke continuous round of hepatocyte damage and regeneration, thus causing chronic liver disease.

The first step towards HCC is the formation of *hyperplastic nodules* of regenerating hepatocytes that have normal cytological features. Then these lesions can progress to *pre-malignant dysplastic nodules,* which have abnormal cytological features including clear cell changes and nuclear crowding. *Pre-malignant dysplastic nodules* may evolve to HCC that is able to invade the surrounding fibrous stroma and vessels and occasionally has metastatic potential.

The molecular analysis of human HCC has shown many genetic and epigenetic alterations that result in the deregulation of key oncogenes and tumor suppressor genes including: TP53, β-catenin, Erb, hepatocyte growth factor receptor (MET) and its ligand hepatocyte growth factor (HGF), p16, E-cadherin and cytochrome c oxidase subunit II (COX2). As a result, HCC arises from a unique combination of somatic genetic alterations in various signalling pathways that cooperate to promote oncogenesis.

Recently, an increasing number of reports have highlighted the interaction of autophagy with these pathways and its dual role in the carcinogenesis, inhibiting the initiation process, while promoting tumor growth, metastasis and therapeutic resistance during tumor progression (Fig.1).

3.1 Protective effect of Autophagy in Hepatocellular carcinoma initiation

Several lines of evidence suggest that autophagy protects against tumor initiation by maintaining intracellular homeostasis. *Atg* genes play a critical role in the induction of autophagy. The deletion of *Beclin1*, *Atg5* and *Atg7* were found to be associated with spontaneous tumorigenesis (Qu, Yu et al. 2003, Komatsu , Waguri et al. 2005, Takamura, Komatsu et al. 2011). The first link between autophagy and cancer development was established with the finding that *Beclin1* inhibits tumorigenesis (Liang, Jackson et al. 2000, Takamura, Komatsu et al. 2011). In fact, it has been shown that the frequency of spontaneous malignancies increases in *Beclin1* $^{+/-}$ mutant, where this mutation accelerate the development of HBV induced pre-malignant injury, together with increased cell proliferation and reduced autophagy *in vivo* (Qu, Yu et al. 2003).

Also, a mouse model with *Atg5* deletion demonstrated the development of liver adenomas, suggesting a tumor suppressive function of autophagy (Takamura, Komatsu et al. 2011). Furthermore, hepatic tumor cells showed swollen mitochondrial, oxidative stress and genomic damage responses. Similarly, *Atg7* $^{-/-}$ deficient mice displayed the same phenotype with the development of liver tumors (Takamura, Komatsu et al. 2011).

The deletion of *Atg5, Atg7* and *Beclin1* leads to accumulation of p62 resulting in the development of hepatocellular carcinoma (Ichimura, Kumanomidou et al. 2008). P62 is an autophagic substrate that is used in measuring autophagic activity. Its knockdown inhibits growth and proliferation. Moreover, autophagy deficiency increases damaged mitochondria accumulation, oxidative stress and deficiency in DNA repair which lead to chronic tissue damage and genome mutations in HPCs (hepatic progenitor cells), two key factors of oncogenesis.

Autophagy has been suggested to prevent cancer progression by suppression of inflammation. This was first observed in autophagic deficient mice showing elevated levels of inflammosome associated IL-1β and IL-18 cytokine production compared to wild type control (Saitoh, Fujita et al. 2008). Autophagy suppression was also associated with high levels of CXCL17 that promotes cell proliferation and migration. Its silencing induces autophagy thanks to the nuclear translocation of liver kinase B1 (LKB1) that phosphorylates and activates AMPK, resulting in the reduction of tumor volume and proliferation (Wang, Li et al. 2019).

Moreover, the protective effect of autophagy involved enhanced degradation of yes associat-

ed protein 1 (YAP), the major nuclear effector of the Hippo pathway that controls liver growth and YAP overexpression (Perra, Kowalik et al. 2014, Lee, Noon et al. 2018). YAP has been identified as an autophagy substrate and as an essential downstream mediator of tissue remodelling, progenitor cell activation and hepatocarcinogenesis in autophagy deficient liver. Indeed, mice with *Atg7* deficiency displayed increased YAP protein levels and overexpression of YAP target genes that drives hepatocyte proliferation leading to gross hepatomegaly. The deletion of YAP in *Atg7*$^{-/-}$animal model reduces HCC incidence. Therefore, autophagy, by controlling the degradation of YAP, acts as a gatekeeper of hepatic differentiation, growth regulation and carcinogenesis(Lee, Noon et al. 2018).

Autophagy has also been shown to contribute to the anti-proliferative activity of interferon gamma (INF-γ) that exerts anti-viral and anti-proliferative effects in cancer cells. Its inhibitory effects are abolished when autophagy is inhibited (Li, Du et al. 2012).

Autophagy can also be modulated by microRNA (miRNA). HCC multiple miRNAs that target autophagic genes can influence tumor growth.(Zhu, Wu et al. 2009, Frankel, Wen et al. 2011, Chang, Yan et al. 2012). For instance, miR-7 is a short non-coding molecule with a well-known tumor suppressive role in different cancer types. In HCC, miR-7 levels are significantly downregulated compared to normal samples. A forced increase of miR-7, causes an increase in autophagic activity by targeting the mTOR pathway. Overall, this leads to a decrease in cancer cell proliferation (Wang, Wang et al. 2017).

miR-85 is an essential component in liver tumor development, acting as tumor suppressor. In HCC cell line HepG2, miR-85 upregulates autophagy activity with a functional effect on cell cycle arrest(Zhou, Liu et al. 2017).

LncRNA PTEN1 (long non-coding RNA-PTEN1), a pseudogene of the tumor suppressor gene PTEN, induces autophagy as a pro-death response to suppress hepatocellular carcinoma (Chen, Tseng et al. 2015). LncRNA PTEN1 prevents the interaction of different miRNA with PTEN that in this way can inhibit the activation of PI3K/AKT pathway, thus inducing pro-death autophagy, resulting in the HCC cells death (Tay, Kats et al. 2011).

All these lines of evidence elucidate that autophagy mediates anti-tumor effects and participates in various signalling pathways directly or indirectly to prevent the onset and progression of hepatocellular carcinoma.

3.2 Autophagy as a pro-cancer mechanism in liver cancer

To date, important insights associate the activation of autophagy with several stress responses including starvation, growth factor deprivation, hypoxia, damaging stimuli and therapeutic agents. The overall functional effect in cancer cells, including HCC, is the activation of a pro-survival mechanism(Chen, Tseng et al. 2015). For example, basal autophagy is elevated in hypoxic regions of some tumor types, where plays an essential role in tumor cell survival. In fact, tumor neovascularization may not result in a homogenous vessels network, especially in the fast-growing tumor, where there are some regions within cancer cells depend on autophagy for their survival, due to the limited nutrients and oxygen (Degenhardt, Mathew et al. 2006). In hepatocyte and HCC, hypoxia induced autophagy through the stabilization of the transcriptional factor HIF (hypoxia inducible factor) that controls oxygen homoeostasis. In hypoxic conditions, the upregulation of HIFα induced autophagy by inhibiting the interaction between BCL-2 and Beclin1. This is due to the upregulation of Bcl-2/adenovirus E1B 19-kDa interacting protein 3 like (BNIP3) protein that by interacting with BCL-2 inhibits BCL-2/Beclin1 binding (Bellot, Garcia-Medina et al. 2009). Furthermore, hypoxia upregulates early growth response gene 1(Egr-1), a zinc finger nuclear protein that functions as a transcriptional regulator, that promotes migration in HCC cell lines (Sijtsema 1977). The first outcome of the hypoxic stress, is the extensive production of reactive oxygen species (ROS) that oxidize cellular components including DNA, lipids and proteins (Bjelland and Seeberg 2003, Scherz-Shouval and Elazar 2007). Tumors activate different mechanisms to eliminate intracellular ROS, like the upregulation of antioxidant protein NRF2 (Jain, Lamark et al. 2010). NRF2 is a cytoplasmic protein, that is

upregulated during autophagy, and translocates into the nucleus to regulate the transcription of different redox-balance proteins. Another way to control the excessive ROS production is by the removal of damaged organelles. Non-functional mitochondria represent the main source of ROS and the induction of mitophagy helps cell in the removing of these damage organelles, to maintain cell functions and bioenergetics(Lemasters 2005).

The analysis of 156 HCC patients has reported the presence of elevated levels of LC3-II (a key autophagic marker) and correlated this overexpression with clinical features including vascular invasion and lymph nodes metastasis. Moreover, the overexpression of LC3-II was also associated with an overall survival rate inferior of 5 years, thus suggesting autophagy involvement in the development and poor prognosis of HCC (Wu, Jia et al. 2014). Indeed, in HCC patients with advanced liver cancer, increased autophagy correlates with low survival rate (Lazova, Camp et al. 2012, Wu, Jia et al. 2014).

In HCC, miR-375 that is known to inhibit autophagy through the downregulation of ATG7, resulted to be under-expressed. Under hypoxic conditions, miR-375 suppressed the conversion of LC3-I in LC3-II thus blocking the autophagy flux, mitophagy in HCC cells and the elimination of damaged mitochondria to impaired viability of HCC cells.

These data suggested that autophagy promotes the survival of HCC cells under hypoxic condition in patients with a confirmed diagnosis of HCC.

In addition, autophagy is known to promote liver cancer development by inhibiting the expression of tumor suppressor (e.g. p53, p16, p21, and p27). As well, .it has been reported that the hypoxia induced autophagy contributes to the chemoresistence of HCC cells. Thus, blocking autophagy may be an ideal target for HCC. Some works have demonstrated the increased anti-cancer efficacy of Sorafenib (the only FDA approved therapy for HCC) when autophagy key genes like *BECN-1* and *ATG5* are inhibited. This data suggest that autophagy inhibitors may have a synergistic anti-tumor effect with chemotherapy (Yuan, Li et al. 2014).

Taken together, all these data support the idea that the pro- tumoral role of autophagy in hepatocarcinoma depend on the stages of the tumor development and that the inhibition of autophagy may be an anti-tumor mechanism in established HCC.

4. Potential therapy targeting of autophagy in hepatocellular carcinoma

The potential therapeutic value of targeting autophagy in hepatocellular carcinoma arises from the consideration that autophagy is an accomplice of cancer cell survival under stress conditions. For this reason, autophagy inhibitors may enhance the sensitivity of cells to hypoxia and metabolic stress.

Several studies have demonstrated, in mouse model, that autophagy inhibition could enhance cell death by promoting the activation of tumor suppressor pathway. Autophagy inhibitors like 3-metyladednine (3-MA), which blocks the fusion between auto-phagosome and lysosome, can increase the effect of a meloxicam, a COX-2 selective drug, that has an anti-tumor effect in different tumors (Zhong, Dong et al. 2015). Similarly, Sorafenib, the earliest approved therapeutic drug for HCC patients with advanced stage of liver tumor, demonstrated an enhanced anti-tumor efficacy when autophagy is inhibited by Chloroquine (CQ), bafilomycin A1, or by a siRNA against *Beclin1* or *Atg5*. Sorafenif acts by targeting the RAF/MEK/ERK pathway, leading to the inhibition of tumor growth and neoangiogenesis (Wilhelm, Carter et al. 2004). However, it has been shown that Sorafenib is also able to induce autophagy both *in vitro* and *in vivo,* thus promoting survival of hepatocellular carcinoma*,* through an ERK/MAPK independent pathway (Shimizu, Takehara et al. 2012).

These different data suggest that the balance of the autophagy mechanism can be a way to overcome cellular resistance towards some antineoplastic treatments.

However, the physiological functions of autophagy are very important for normal cells and tissues. For this reason, an important question that remains open is whether a systemic autophagy defect affects only cancer growth or also normal tissue. Thus, several challenges will have to be addressed before testing autophagy modulating-approaches in clinical trials (Levine and Kroemer 2019). Firstly, it is necessary a better characterization of autophagy related

pathways in liver diseases; in particular, it should be consider that ATG genes can be involved in non-canonical autophagy pathway and that the manipulation of autophagy can interfere with other interconnected pathways. Secondly, it is very important to discover autophagy biomarkers to follow *in vivo*, for the development of autophagy-targeted strategies.
Thirdly, it is necessary to define a therapeutic time-window for chronic liver diseases, since type and level of autophagy changes during the progression of the disease. Finally, we need the development of strategies that target a specific type of cells in the liver, considering the important role of autophagy in all organs.
Therefore, all these aspects represent a great hotspot and a breakthrough point for reducing HCC risk and improving therapeutic efficacy.

5. Conclusions and future perspectives

Since the identification of the first ATG genes in yeast in 1990, significant efforts have been made in the understanding of molecular mechanisms driving autophagy. One of the emerging fields is the involvement of autophagy in cancer development. Among different kinds of cancer, HCC appears to be relevant to autophagy. In HCC, autophagy seems to play a dual role: it acts as tumor suppressor in the initial step of the disease, while promotes tumor development once HCC is well established. Therefore, different approaches are needed to modulate autophagy for liver cancer prevention and therapy. Although the high potential for autophagy modulation as a therapeutic method for HCC, the clinical application of these autophagy modulators remains unclear.
To summarize, future studies on the various functions of autophagy according to tumor stage, differentiation, and environmental and genetic factors are needed for the development of new treatment options for HCC patients.

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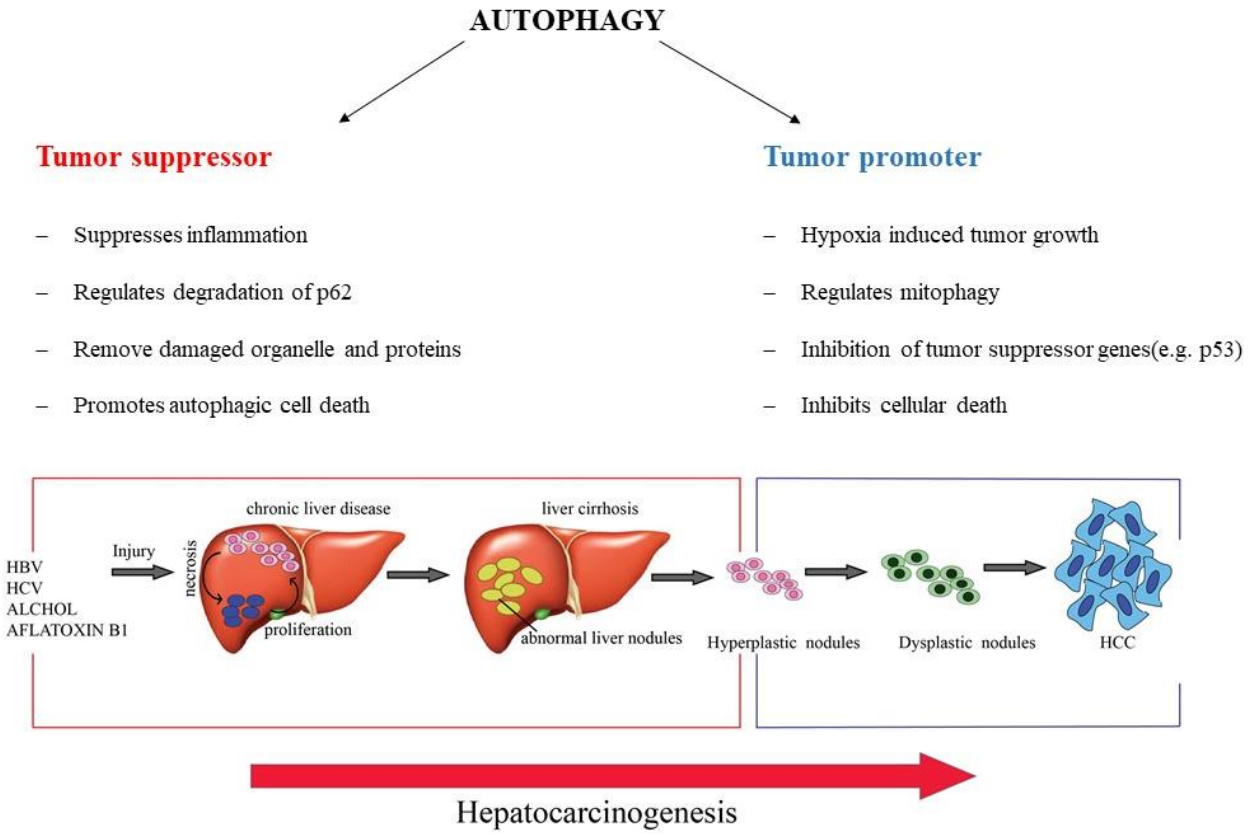

Figure 1: Dual role of autophagy in the initiation and development of hepatocellular carcinoma.
Hepatic autophagy is activated by various factors (HBV and HCV viral infections, heavy alcohol consumption, aflatoxin B1 contaminated food) that can cause hepatic injury after which there is necrosis followed by hepatocyte proliferation. Continuous cycles of this destructive–regenerative process promotes a chronic liver disease condition that culminates in liver cirrhosis. Subsequently, there is the formation of hyperplastic nodules, followed by dysplastic nodules and ultimately hepatocellular carcinoma (HCC) development.
By limiting inflammation, P62 accumulation, oxidative stress response and consequently inhibiting genomic instability, autophagy can serve as a tumor suppressor in the initiation stage of hepatocarcinogenesis. On the other hand, autophagy acts as a pro-survival mechanism to protect liver cancer cells against cell death induced by hypoxia, oxidative stress, starvation, DNA damage and therapeutic stress, thus promote liver cancer development

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