Autophagy Modulation in Cholangiocarcinoma

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Autophagy is a multistep catabolic process through which misfolded, aggregated or mutated proteins and damaged organelles are internalized in membrane vesicles called autophagosomes and ultimately fused to lysosomes for degradation of sequestered components. The multistep nature of the process offers multiple regulation points prone to be deregulated and cause different human diseases but also offers multiple targetable points for designing therapeutic strategies. Cancer cells have evolved to use autophagy as an adaptive mechanism to survive under extremely stressful conditions within the tumor microenvironment, but also to increase invasiveness and resistance to anticancer drugs such as chemotherapy.

cholangiocarcinoma autophagy inhibition autophagy activation chemoresistance

1. Introduction

Cholangiocarcinoma (CCA) is a very aggressive epithelial cell malignancy arising from varying locations within the biliary tree, a complex network of ducts that deliver bile to the gallbladder and to the intestine [1]. CCA originates from cholangiocytes located at any portion of the biliary tree and represents the most common biliary duct malignancy and the second most frequent cancer of the liver after Hepatocellular Carcinoma (HCC), accounting for 10–20% of all primary liver cancers [2][3][4].

The classification of CCA has been a matter of debate during the past decades, and depending on different aspects of these tumors, several classifications have been proposed. Based on the anatomy of the biliary tract and the different origins of the tumor, CCA is classified into three different types: intrahepatic cholangiocarcinoma (iCCA), which originates from the biliary tree within the liver proximal to the second-order bile ducts; and extrahepatic cholangiocarcinoma (eCCA), which originates outside the liver parenchyma. eCCA is further subdivided into perihilar cholangiocarcinoma (pCCA), arising between the second-order bile ducts and the insertion of the cystic duct into the common bile duct; and distal cholangiocarcinoma (dCCA), arising between the insertion of the cystic duct and the ampulla of Vater [2][5][6]. Although this anatomical classification is the most widely used, other factors such as tumor growth pattern (mass-forming, periductal infiltrating or intraductal) and the cell of origin (cholangiocytes, peribiliary glands, hepatic progenitor cells or hepatocytes) offer alternative classification that may be more useful in specific clinical settings [7][8][9][10].

CCA is a very deadly cancer which at an early stage remains asymptomatic and is normally diagnosed at advanced stages and in the elderly, where therapeutic options are reduced and have limited efficacy, showing high chemoresistance and death rates [2][11][12]. The only curative treatment is radical surgical resection and liver

transplantation, which are limited to curing locally restricted disease [13][14]. However, most newly diagnosed patients present with advanced or even metastatic stages of disease, and chemotherapy is the only treatment option. Among all chemotherapeutic regimes available, only the combination of gemcitabine and cisplatin exerts some growth-inhibiting effects at advanced stages of the disease [15][16].

Autophagy is a multistep self-degradative cellular process in which misfolded, aggregated or mutated proteins and damaged organelles such as mitochondria, endoplasmic reticulum (ER) or peroxisomes are sequestered in double-membrane vesicles, which fuse with lysosomes for further degradation [17][18]. This tightly regulated process is important for maintaining nutrient and energy homeostasis and eliminating intracellular pathogens. Giving the housekeeping function of autophagy, it is generally a survival mechanism, but due to the multistep condition of the process and the multiple control points, autophagy can be deregulated at multiple sites, leading to multiple human diseases, including cancer [19]. Autophagy has been shown to act as a tumor promoter as well as a tumor suppressor in cancer, depending on the cell context, and autophagy modulation has arisen as a promising therapeutic strategy to treat cancer [20][21][22][23][24][25]. Even though the molecular mechanisms of autophagy regulation of tumor biology are not fully understood, multiple reports are showing promising therapeutic potential in combination with other drugs, such as chemotherapy [26].

2. Cholangiocarcinoma Genetic and Epigenetic Alterations and Autophagy

CCA is a very heterogeneous group of malignancies highly influenced by different risk factors and genetic and epigenetic alterations [27]. Surgery, chemotherapy and locoregional therapy are the only approved therapies for CCA, although less than one-third of the patients have been classified as having a resectable tumor at the time of diagnosis. Tumor resection is usually followed by adjuvant chemotherapy using gemcitabine, cisplatin or 5-FU (5-fluorouracil), which nevertheless does not prevent the high rates of relapse and resistance. For patients presenting with unresectable or metastatic CCA, systemic chemotherapy remains the mainstay palliative treatment modality, and only gemcitabine plus cisplatin combination has offered limited advantages [15][16], usually followed by a fluoropyrimidine-based regimen when gemcitabine-based treatment fails [27]. The identification of genetic and epigenetic alterations and the increased knowledge about the molecular pathophysiological mechanisms governing cholangiocarcinogenesis and tumor recurrence, resistance and metastasis have allowed the development of more specific therapies, although clinical results evaluating specific molecular agents demonstrate no or only very modest survival benefits of the agents tested [4][5][28].

Whole-genome analyses identified two distinct genomic classes of iCCA: an inflammatory class with predominant activation of inflammatory pathways, and a second proliferative class with predominant activation of oncogenes that correlate with worse patient outcome [29]. Next-generation sequencing analysis revealed that the majority of CCAs showed a driver gene mutation, although tumors from different sites (iCCA versus pCCA and dCCA) have different genetic profiles. For example, RAS appears frequently mutated in CCA, with a higher prevalence in dCCA [30]. Exom sequencing analysis identified a unique subtype of CCA without RAS mutation and/or FGFR2 fusion genes [31]. Epigenomic studies have revealed that epigenetic modification such as DNA hypermethylation, histone

modifications and microRNAs deeply affects CCA development [32]. All these data support the complexity of this type of cancer and the low efficacy of current diagnostic methods and therapies, and deeper research into the mechanisms leading to CCA establishment and progression will help to support the development of novel treatments that could improve therapeutic outcome based on proper patient classification.

Chronic inflammation, partial bile flow obstruction (i.e., cholestasis) and bile duct injury are recognized to be major features for malignant transformation [33]. Upon chronic inflammation, both cholangiocytes and immune cells secrete pro-inflammatory cytokines such as IL-6, endotoxins or TNF-α. Sustained IL-6 production acts as a key player in hepatobiliary inflammation and cancer development, promoting mitogenic responses and cell survival [34]. Additionally, IL-6 can increase nitric oxide synthase (iNOS)-mediated nitric oxide production, resulting in DNA damage [35] and cyclic oxygenase (COX)-2-mediated prostaglandin secretion that results in cell growth, antiapoptosis and angiogenesis [36]. Autophagy plays a relevant role in inflammation, although understanding of this interconnection is still incomplete [37]. Many of the signaling pathways that control inflammation during tumorigenesis are also known regulators of autophagy. For example, in lung cancer cells exposed to arsenic, oncogenic transformation correlates with sustained upregulation of IL6 and reduced autophagy [38], and IL-6-dependent transformation requires inhibition of a Beclin1-Bcl2 complex, which is dependent on STAT3 signaling. Moreover, enhancement of autophagy via Beclin1 overexpression is sufficient to block IL-6 mediated transformation [38]. This correlation between IL-6-mediated carcinogenesis and autophagy may represent an interesting and promising approach to treat iCCA with an inflammatory component. Additionally, there are a large number of studies that relate different pro-inflammatory pathways with ER stress and autophagy [37][39][40].

To date, different genes have been related to cholangiocarcinogenesis. Activating KRAS mutations can be found in up to 40% of CCAs, with major prevalence in dCCA and associated with a worse prognosis [30]. In a small study on 54 clinical samples of iCCA, 7.4% of cases were KRAS mutated and associated with higher tumor stage and worse long-term overall survival, as well as a greater likelihood of lymph node involvement [41]. Moreover, in a murine model of iCCA development harboring KRAS mutation and p53 inactivation, two of the most common genetic alterations in CCA [30][42], KRAS mutation collaborates with p53 deletion to cause hepatic transformation and reduced survival [43]. This murine model recapitulates histopathologic features of human iCCA and shows high basal levels of autophagy associated with tumor growth. Inhibition of autophagy with chloroquine (CQ) inhibited the growth of these cells and accumulated LC3-II, indicative of an active autophagy directly involved in tumor progression [43]. This data correlates with human iCCA cell lines mutated in KRAS and with p53 deficiency, which show elevated autophagy compared with normal iCCA cells, and CQ also inhibited the growth of these cells [44], similar to the situation described for pancreatic and lung cancers [45][46][47][48][49][50]. No specific RAS inhibitors have been developed so far, and targeted therapies aiming to modulate KRAS downstream pathways such as MEK1/2 inhibitor selumetinib are in development for CCA, pointing to the potential combination with autophagy inhibitors to improve their therapeutic potential [4][51].

Alterations in c-MET, the overactivation of which leads to activation of MAPK, PI3K/Akt and STAT pathways, correlates with high grade, invasiveness and poor prognosis in CCA [52][53], and its inhibition promoted autophagy in lung cancer cells [54], further linking c-MET-mediated autophagy inhibition in carcinogenesis. The gain of function

mutation in ERBB2 and EGFR genes correlates with malignancy in human cholangiocytes, cancer progression and poor survival [55][56], and treatment with tyrosine kinase inhibitors induced protective autophagy in different cancer types [57], suggesting that the combination with autophagy inhibitors could increase the efficacy of these compounds. Similarly, FGFR2 fusion genes that result in altered cell morphology and increased cell proliferation have been described in CCA [58]. It has been shown that FGFR alterations suppress autophagy, which could be associated with initial steps of carcinogenesis, and genetic or pharmacological FGFR inhibition in vitro induces protective autophagy in lung and breast cancer; therefore, inhibition of autophagy increases anticancer efficacy of FGFR inhibitors in these cells [59][60]. There are currently FGFR inhibitors in clinical development for CCA, opening the possibility of evaluating the combination of these inhibitors with autophagy modulators to increase efficacy. Loss of SMAD4 is also frequently observed in CCA in the distal common bile duct [61], and it has also been shown to render pancreatic cancer radioresistance through promotion of autophagy [62]; hence, a combination with autophagy inhibitors also could potentially apply to these mutated tumors. Adenomatous Polyposis Coli (APC) is an additional tumor suppressor commonly mutated in CCA and may be responsible for the early stages of carcinogenesis [63], stages where dysfunctional autophagy has also been detected in clinical samples [64] and in xenografts during tumor formation [65].

Additionally, it has been proposed that epigenetic changes such as histone modifications, DNA methylation and noncoding RNAs, which play a very relevant role in the pathophysiology of CCA [66], are also regulators of autophagy [67]. Overexpression of histone deacetylase 6 (HDAC6) was reported in CCA, promoting the shortening of the primary cilium and inducing hyperproliferation. HDAC6 inhibition restores ciliary expression and decreases tumor growth in CCA [68][69], a mechanism that has been shown to be mediated by autophagy inhibition in colorectal cancer, multiple myeloma and neuroblastoma [70]. Other HDACs, such as HDAC1, have been found overexpressed in CCA and correlate with malignant behavior and poor iCCA prognosis [71]. Histone methylations also control autophagic flux, and it has been proposed that histone methylation keeps the brakes on autophagy [72]. DNA-methylation-mediated silencing of tumor suppressor genes is often seen in CCA. Frequent mutations in both DNA methylation IDH1 and IDH2 have been reported in 10% of iCCA, which are associated with hypermethylation of CpG shore, resulting in an altered state in the cellular process of differentiation [73][74]. Several reports highlight the link between autophagy inhibition and histone methylation [67][72], suggesting autophagy inhibition as a target for treating IDH mutant gliomas [75]. A number of microRNAs (e.g., miR-141, miR-200b, miR-21, miR-29b among others) have been described to be either up- or downregulated in CCA cell lines, and their predicted targets were found to be associated with cell growth, apoptosis and response to chemotherapy in CCA cell lines [76][77]. MicroRNAs are also involved in regulating autophagy in cancer, and different autophagy-related proteins have been described as miRNAs targets, such as ULK2, Beclin1, LC3, ATG4 and ATG9 [78][79]. Moreover, miR-124 has been described to induce cytotoxic autophagy in CCA through the EZH2–STAT3 pathway in vitro and in vivo [80].

3. Autophagy Modulation in Cholangiocarcinoma

Although the pathologic role of autophagy in cholangiocarcinogenesis and the therapeutic potential of its modulation are still poorly understood, several reports have identified autophagy-related markers with prognostic

significance, underlining the relevance of this process in CCA and offering novel therapeutic avenues.

Similar to pancreatic cancer, CCA follows a carcinogenic development in which a precursor lesion, a biliary intraepithelial neoplasia (BilIN), is developed. The study of the expression levels of LC3, Beclin1 and p62, along with p53 and KRAS status on clinical BilIN samples and compared with normal bile duct and peribiliary gland, revealed that autophagy deregulation may occur at an early stage of development of CCA [64]. Expression of LC3 and p62 was high in BilIN stages 1-2 compared with normal cholangiocytes, and LC3, Beclin1 and p62 were all higher in invasive carcinoma compared with nontumoral tissue. No significant correlation between KRAS and expression of autophagy markers in BilIN 1-2 stages was observed. Autophagy is a dynamic process, and accumulation of LC3-II and p62 in initial steps of cholangiocarcinogenesis could reflect a defect in the later processing of autophagosomes rather than increased rates of autophagy. This would correlate with the tumor suppressor role of autophagy in these initial steps, where its inhibition could permit carcinogenic transformation of cholangiocytes.

Epithelial to Mesenchymal Transition (EMT) is considered to be a major driver of cancer exacerbation, promoting tumor progression, metastasis and drug resistance [81][82]. The link between EMT and autophagy has been amply demonstrated, since main pathways regulating autophagy have a dramatic impact on EMT, such as PI3K/AKT/mTOR, Beclin1, p53 and JAK/STAT signaling pathways. Additionally, signaling pathways implicated in EMT are crucial in autophagy, including integrins, WNTs, NF-kB, and TGF-β signaling pathways [83]. In CCA, EMT leads to immunosuppression through SNAIL expression [84] and is critical for invasiveness and metastasis induced by TGF-β1/SNAIL activation [85]. Autophagy inhibition with CQ reduced invasive capacity under starvation and in TGF-B1-induced CCA cell invasion [86], further exposing EMT and autophagy relation in CCA and reinforcing the idea of a tumor promoter role of autophagy in established CCA tumors.

Beclin1 plays a relevant role linking autophagy, apoptosis and differentiation, and its inactivation and consequent deficiency in autophagy was correlated with malignant transformation, although existing data on the prognostic role of Beclin1 in human carcinomas is contradictory, appearing under- and overexpressed in distinct human cancers [87][88][89]. Several studies have shown the significance of Beclin1 in iCCA [90][91] and eCCA [91], revealing its potential prognostic value for CCA. Beclin1 was found markedly expressed in iCCA samples compared with normal bile duct epithelium [90], and among Beclin1-positive samples, those with low Beclin1 expression were significantly associated with lymph node metastasis, worse overall survival and less disease-free survival [90][91]. Moreover, in a lymph-node-negative CCA subgroup, Beclin1 was higher than in the lymph-node-positive subset, suggesting that Beclin1 inactivation and therefore impaired autophagy might promote malignant phenotypes. Interestingly, a stratified survival analysis in patients with Beclin1 low expression, iCCA patients showed a worse overall survival and progression-free survival than eCCA [91], which may indicate a higher implication of autophagy in iCCA subgroup of patients. Nevertheless, low Beclin1 levels show a correlation with poor prognosis in both subtypes [91]. This clinical data is in contradiction with other reports that indicate an exacerbated autophagy in CCA samples and its association with lower survival and tumor dissemination. Ambra1, a positive regulator of the Beclin1-dependent program of autophagy, positively correlated with SNAIL expression in CCA patients. SNAIL is a hallmark of EMT activation, which is in accordance with the in vitro increased invasive potential mediated by autophagy in TGF-

β1/SNAIL-induced EMT [86]. These opposing results underscore the need to clearly define the type of studies that would help to discern whether the presence of autophagy-related markers are associated with impaired or increased autophagic flux, and additional expression studies of other markers such as LC3-II, p62, PI3Ks or ATGs could add significant value.

In another recent study, Chen and colleagues demonstrated for the first time that LC3B is an independent biomarker for overall survival and progression-free survival in iCCA patients, and that high LC3B staining significantly associates with poor tumor differentiation, tumor stage, early relapse and bad long term survival. Based on nomograms, they stratified iCCA patients and generated a therapeutic strategy after hepatectomy, demonstrating that nomograms based on autophagy markers can be considered as effective models to predict postoperative survival of iCCA patients [92]. In a very interesting study published in 2019, Atg7 was found to be a causative genetic risk factor for CCA development in a family with a high incidence of pCCA, identifying a germline mutation associated with CCA development [93]. This genetic variant resulted in the accumulation of p62, indicative of impaired autophagy in the tumors of carriers compared with noncarrier tumors, confirming autophagy pathway perturbation as a novel cancer driver mechanism in human tumorigenesis in correlation with the detection of impaired autophagy in BilIN lesions [64].

Another potential therapeutic target associated with autophagic flux in CCA is FOXO1. FOXO1 expression and transcriptional activity are involved in promoting cellular autophagy, and the interaction of acetylated FOXO1 with ATG7 regulates basal and starvation-induced autophagy in CCA cells [94]. Cytoplasmic accumulation of FOXO1 is associated with increased proliferation in cholangiocytes [95] and pharmacological inhibition of acetylated FOXO1, which results in autophagy inhibition, leads to apoptosis induction and reduced viability of CCA cells [94]. Epigenetic alterations are frequent in CCA, such as miR-124, which was found significantly downregulated in the tumor tissue of patients and in CCA cell lines, and its administration in vitro induced cytotoxic autophagy in CCA cells [80], supporting a protumoral role of epigenomic-mediated inhibition of autophagy.

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