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TOPIC HIGHLIGHT

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Nervous and Neuroendocrine regulation of the pathophysiology of cholestasis and of biliary carcinogenesis

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Abstract

Cholangiocytes, the epithelial cells lining the biliary ducts, are the target cells in several liver diseases. Cholangiopathies and cholangiocarcinoma generate interest in many scientists since the genesis. The developing mechanisms, and the therapeutic tools of these diseases are still undefined. Several studies demonstrate that many hormones, neuropeptides and neurotransmitters regulate malignant and non-malignant cholangiocyte pathophysiology in the course of chronic biliary diseases. The aim of this review is to present the findings of several studies published in the recent years that contributed to clarifying the role of nervous and neuroendocrine regulation of the pathophysiologic events associated with cholestasis and cholangiocarcinoma development. This manuscript is organized into two parts. The first part offers an overview of the innervation of the liver and the origin of neuroendocrine hormones, neurotransmitters and neuropeptides affecting cholangiocyte function and metabolism. The first section also reviews the effects played by several neuroendocrine hormones and nervous system on cholangiocyte growth, survival and functional activity in the course of cholestasis. In the second section, we summarize the results of some studies describing the role of nervous system and neuroendocrine hormones in the regulation of malignant cholangiocyte growth.

Key words: Cholangiocyte; Neuroendocrine hormones; Neurotransmitters; Neuropeptides; Cholestasis; Nervous System; Biliary carcinogenesis; Pathophysiology; Cholangiocarcinoma; Proliferation

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INTRODUCTION

Cholangiocytes are the epithelial cells that line the intrahepatic biliary tree. Their physiologic role is to modify the bile of canalicular origin, through a wide array of absorbtive and secretive processes[1,2]. Indeed, although in normal conditions they represent approximately the 4%-5% of the liver mass, they contribute for at least the 10%-30% of the bile flow[1].

Cholangiocytes are the target of chronic diseases, termed cholangiopathies[3,4], that are commonly characterized by dysregulation of the balance between cell growth and survival. In the course of such diseases there is impaired proliferative response to duct injury and increased cell death by apoptosis, that leads to vanishing of bile ducts[3] and functional liver failure at the end stage. Cholangiopathies thus represent a daily challenge for the clinicians, since definitive medical treatments are not available yet. As a consequence, 20% of liver transplants among adults and 50% of those among pediatric patients are due to these disorders[5].

The malignant transformation of cholangiocytes gives origin to cholangiocarcinoma[6]. Cholangiocarcinoma is often diagnosed at late stages; its surgical resection has given very limited success, as response of this neoplasm to conventional chemotherapy[6] is minimal. These features make cholangiocarcinoma a malignancy characterized by a very poor prognosis. In addition, the incidence and prevalence of cholangiocarcinoma are increasing worldwide[7].

Many of the problems that arise in the management

of cholangiopathies and of cholangiocarcinoma can at least in part be ascribed to the poor knowledge about their pathophysiology. Very little is in fact known on which factors are able to affect cholangiocyte biology, with particular regard to those endogenous molecules or systems that affect either cholangiocyte proliferation, survival or functional activity[4].

Bile acids are endogenous factors that have been historically investigated more in this regard, and their properties in modulating cholangiocyte biology and cholangiocarcinoma development are now unanimously accepted[8-13]. More recently, several studies demonstrated that many hormones, neuropeptides and neurotransmitters are able to affect malignant and non malignant cholangiocyte biology as well. This data showed that the biliary epithelium is similar to the gastric or intestinal epithelia, for which the key role played by nerves and neuroendocrine hormones has been known for years[14]. Moreover, it has been shown that in the course of chronic cholestasis, cholangiocytes acquire a neuroendocrine phenotype, that is not proper for these cells in normal conditions[15]. Immunohistochemical studies have also reported that many biliary tract carcinomas (up to the 70% of the cases) show a neuroendocrine differentiation[16,17].

The purpose of this review is therefore to summarize the evidences from the several studies published in the recent years that contributed to clarify the role of nervous and neuroendocrine regulation of the pathophysiologic events associated with cholestasis and cholangiocarcinoma development. In particular, we will focus on how these factors affect cholangiocyte cell biology. We suggest those who might be interested to learn the effects of nerves and neuroendocrine hormones on other liver cell type biology to other reviews recently published on this subject[18-20].

ORIGIN OF NEUROENDOCRINE HORMONES, NEUROTRANSMITTERS AND NEUROPEPTIDES AFFECTING CHOLANGIOCYTES

Neuroendocrine hormones are secreted by neuroendocrine cells, that are diffusely present in the whole gastrointestinal tract, in particular the stomach, small bowel[21], as well as pancreas[22]. If these cells are considered the main source of the neuroendocrine hormones, in this review some studies suggest that cholangiocytes them-selves can synthesize some of these peptides in the course of cholestasis.

The liver is innervated by both sympathetic and parasympathetic nerves, whose fibers are located around the hepatic artery, portal vein, intrahepatic and extrahepatic bile ducts[23,24]. Sympathetic nerves originate from the celiac ganglion, whereas the parasympathetic from the vagus nerve[23,24]. Besides catecholamines and acetylcholine, autonomic fibers that innervate the liver can also release other neurotransmitters, like neuropeptide Y (NPY)[25,26], calcitonin gene related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP), enkephalin and bombesin[27-30]. Nerve terminations mostly follow the vascular structures of the portal tract, and have been identified around bile ducts and vessels. Many of the above mentioned neurotransmitters have been shown to modulate intrahepatic hemodynamics[31-33].

Table 1 Neuroendocrine hormones affecting cholangiocyte biology

Hormone	Receptor	Effect on cholangiocyte biology	Reference
Secretin	SR	Stimulates HCO_3^- secretion through the increase of cAMP/PKA and opening of CFTR	1, 34-43
Somatostatin	$SSTR_2$	Counteracts the effect of secretin; stimulates bile absorption	44, 45
Insulin	IR	Counteracts the effect of secretin	46
ET-1	ET_A-ET_B	Counteracts the effect of secretin; reduces the SR expression	47
VIP	unspecified	Increases HCO_3^- and water secretion	48, 49
Bombesin	unspecified	Increases HCO_3^- and water secretion	50
Gastrin	CCK-B/ gastrin	Counteracts the effect of secretin; reduces cell proliferation	40, 51
Estrogens	ERα-ERβ	Stimulate cell proliferation	52-55
Serotonin	$5HT_{1A}$-$5HT_{1B}$	Synthesized and secreted by cholangiocytes in the course of cholestasis. Counteracts the effect of secretin and reduces cell growth	58
GH/IGF-1	GH-R-IGF-1R	Stimulate cell proliferation	59

NERVOUS AND NEUROENDOCRINE REGULATION OF THE PATHOPHYSIOLOGY OF CHOLESTASIS

Regulation of cholangiocyte growth, survival and functional activity by neuroendocrine hormones (Table 1)

The close link between cholangiocytes and neuroendocrine hormones has its basis on the early studies that showed the hormone secretin as the most potent regulator of cholangiocyte functional activity[34,35]. In 1988, Alpini *et al* demonstrated that in a rat model of cholestasis (induced by bile duct ligation, BDL) the infusion of secretin was associated with a marked increase of the bile flow and bicarbonate biliary excretion[35]. After that "landmark" manuscript, a large series of investigations later defined the intracellular mechanisms by which secretin induces such a potent functional stimulus. It is now known that secretin stimulates ductal secretion[1,34-36] by selective interaction with secretin receptors, expressed only by cholangiocytes in rat liver[37]. The interaction of secretin with its own receptors[37] leads to an increase in intracellular cAMP levels[1,36,38-40], activation of PKA[41], opening of CFTR Cl^- channels[42] with activation of the Cl^-/HCO_3^- exchanger[1,36,41,43], which leads to secretion of bicarbonate into bile[35].

Other neuroendocrine hormones were then discovered to affect cholangiocyte choleretic activity. One of the first molecules studied was somatostatin. It was demonstrated that cholangiocytes express the $SSTR_2$ receptor; the interaction of somatostatin with this receptor markedly diminished the effect of secretin on the biliary excretion

of water and bicarbonate by cholangiocytes in cholestatic conditions[44]. Such an effect of somatostatin was due to the fact that the activation of the $SSTR_2$ receptor prevented the increase of the adenylyl-cyclase elicited by secretin[44]. In later studies conduced in IBDUs isolated from wild type and $SSTR_2$-knock out mice, it was also found that somatostatin not only reduces cholangiocyte choleresis, but it also stimulates ductal bile absorption[45].

Cholangiocytes also express at the apical pole the receptor for insulin[46]. Upon its activation, a marked reduction of the secretin-induced choleresis was observed, both if the hormone was administered *in vivo* to BDL rats and if microinjected into the lumen of IBDUs isolated from animals with cholestasis. It was observed that the activation of the insulin receptor determined a cascade of intracellular events that resulted in the inhibition of secretin-stimulated cAMP and PKA activity. Such a chain of events seemed to have its core event in the enhancement of the intracellular Ca^{2+} levels and the consequent activation of the Ca^{2+}-dependent PKCα[46]. An effect similar to the one of insulin has been described for endothelin-1 (ET-1), which interacts with the specific receptors expressed in the biliary epithelium (ET_A and ET_B) and blunts the secretin-induced choleresis of the BDL rat and reduces the expression of the secretin receptor on cholangiocytes[47].

In contrast to somatostatin and insulin, vasoactive intestinal polypeptide (VIP) and bombesin have been found to be able to enhance cholangiocyte choleresis. Both the hormones induced a potent fluid and bicarbonate excretion in IBDUs, but not in hepatocytes, isolated from normal and cholestatic rats. Interestingly, neither VIP nor bombesin had any significant effect on modulating intracellular cAMP levels[48-50]. Detailed pH studies indicated that the underlying intracellular mechanism, at least for bombesin, is its ability to stimulate the activity of Cl^-/HCO_3^- exchange in association with a counterbalancing secondary activation of electrogenic Na^+/HCO_3^- symport[50].

In more recent studies, increasing evidence regarding the ability of neuroendocrine hormones to affect cholangiocyte growth, and functional activity, hvee been reported.

If the infusion of gastrin to BDL rats is associated with the reduction of the choleretic response to secretin by cholangiocytes[40], its chronic administration through an intraperitoneal minipump resulted not only in reduced functional activity, but also in a marked decrease of the bile duct mass[51]. Cholangiocytes indeed express the CCK-B/gastrin receptors[51], which, upon activation, elicit the intracellular Ca^{2+} release, the increase of IP_3 levels, and the membrane translocation (e.g. activation) of the Ca^{2+}-dependent PKCα[51]. In turn, the gastrin-activated PKCα, as above mentioned, is able to interfere with the secretin signaling and modulate the adenyl-cyclase activity, thus reducing the intracellular cAMP levels and PKA activity. These observations, demonstrated that bile acids also activate this intracellular pathway to modulate cholangiocyte growth[8], contribute to elucidate which intracellular signalings sustain the proliferative response of cholangiocytes to cholestasis and by which mechanisms it is possible to modulate them. Moreover, since cAMP/PKA resulted the key molecules implicated in cholangiocyte proliferation, this helped to explain the association between increase of duct mass and enhanced biliary choleresis[8,35].

A major contribution to this field of research has been given by the studies that clarified the effects of estrogens on the biliary epithelium. Both the estrogen receptor (ER) α and β were observed in cholangiocytes[52], their expression being up-regulated after BDL[53]. When cholangiocytes were stimulated *in vitro* with 17-β-estradiol, their proliferation was markedly increased, as a consequence of the ER-dependent activation of Src/Shc/ERK1/2 intracellular pathway[54]. To demonstrate the physiological and pathophysiological relevance of estrogens on cholangiocyte proliferative response to cholestasis, when BDL male rats were treated *in vivo* with antiestrogens like tamoxifen or Ici 182 780[52] or when BDL female rats were subjected to ovariectomy[53], the growth of the biliary tree was blunted and the biliary epithelium underwent programmed cell death by apoptosis[52,53]. This evidence suggested that estrogens are required for a separative response of the biliary tree to injury. These studies produced in the last few years by Alvaro represent a significant change in the knowledge of the role played by the neuroendocrine system on the biliary cell biology. Indeed, primary biliary cirrhosis (PBC), the most common from of cholangiopathies[4], is much more frequent in women than in men, and has its clinical outcome typically after menopause, when the endogenous estrogen levels suddenly drop[55]. Therefore, these observations by Alvaro seem to provide the biological confirmation of the role of estrogens in the progression of PBC, a role that was, earlier, only hypothesized on the basis of epidemiological data. To further support this concept, Alvaro also demonstrated that the ER expression in cholangiocytes is markedly reduced in late stages of PBC[55]. Altogether, Alvaro's studies made clear that gaining knowledge on the role of neuroendocrine hormones on cholangiocyte biology might also be an effective strategy to further understand the pathophysiology of the cholangiopathies and thus eventually to design novel therapeutic strategies.

The neuroendocrine hormone serotonin has been hypothesized to be involved in the genesis of certain clinical features of PBC, like fatigue and pruritus[56,57]. Interestingly, it has been recently shown that cholangiocytes express the serotonin 1A and 1B receptors[58]. When they are activated by selective agonists, the proliferation of BDL cholangiocytes are dramatically reduced, both *in vivo* and *in vitro*[58], in association with the loss of the response to secretin of the markers of cholangiocyte functional activity[58], like the bile flow, the bicarbonate excretion and the intracellular cAMP levels[34]. Such an effect seems more to be mediated by the cross-talk between the Ca^{2+} and the cAMP/PKA signalings. If the serotonin 1A and 1B receptors are activated, there is an increase of Ca^{2+}, IP_3 and PKCα levels, with the consequent reduction of the intracellular cAMP levels and PKA activity. Most interestingly, it was observed that hyperplastic cholangiocytes isolated from BDL rats are able to synthesize and secrete serotonin.[58] If, *in vitro* or *in vivo*, cholangiocyte-secreted serotonin is neutralized,

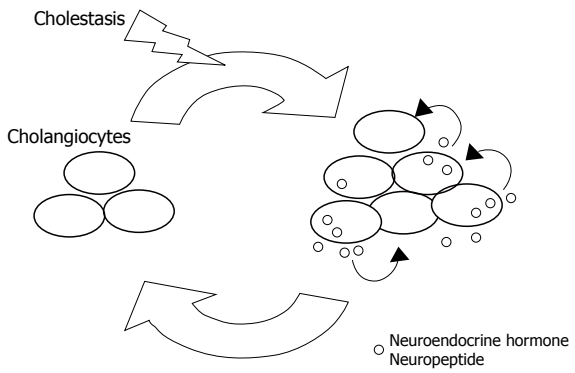

Figure 1 Proposed working model for the existence of an autocrine/paracrine loop of neuroendocrine hormones and neuropeptides that regulate the proliferative response of cholangiocytes to cholestasis. A chronic cholestatic condition induces cholangiocyte proliferation and their transdifferentiation in neuroendocrine-like cell. This allows the biliary epithelium to start to synthesize and secrete a number of peptides that aim to counterbalance the effects of cholestasis itself on cell growth, as a sort of negative feed-back.

Table 2 Neurotransmitters/neurotrophins affecting cholangiocyte biology

Peptide	Receptor	Effect on cholangiocyte biology	Reference
Acetylcholine	M3	Potentiates the effect of secretin. Required for cholangiocyte response to BDL: sustains cholangiocyte proliferation and prevents apoptosis	43, 60, 61, 64, 65
Epinephrine/ Norephinephrine	α_1	Potentiates the effect of secretin	67
Epinephrine/ Norephinephrine	β_1-β_2	Required for cholangiocyte response to BDL: sustains cholangiocyte proliferation and prevents apoptosis	70
Dopamine	D2	Inhibits secretin-induced ductal secretion	73
NGF	TrkA	Secreted by hyperplastic, cholestatic cholangiocytes; sustains the rpoliferative response to BDL	74

cholangiocyte proliferation in response to cholestasis further increases[58]. This study, therefore, proposes an additional novel working model (as shown in Figure 1): in the course of cholestasis, cholangiocytes acquire a neuroendocrine phenotype that allow them to synthesize some neuroendocrine hormones, like serotonin. Once secreted, these hormones aim to counterbalance the excessive proliferative response of the biliary epithelium. It is thus possible to postulate the existence of an autocrine/paracrine loop of peptides and neuroendocrine hormones that modulate with a negative (or an eventually positive) feed-back the response of cholangiocytes to liver injury[58]. The investigation of the presence of other peptides and hormones that are likely to participate with serotonin in this autocrine/paracrine loop might thus be needed to understand certain still obscure clinical aspects of cholangiopathies.

Recent evidence demonstrated that a "stimulatory" autocrine/paracrine loop should also exist. It has been found that cholangiocytes are the target of the growth hormone (GH)-insulin like growth factor (IGF)-1 axis[59]: GH induces IGF-1 expression and release in isolated cholangiocytes, with the consequent stimulation of cell growth by IGF-1[59].

Regulation of cholangiocyte growth, survival and functional activity by neurotransmitters (Table 2)

A number of studies showed that cholinergic nerves regulate bile secretion[60,61]. In bile-fistula dogs with interrupted enterohepatic circulation, distal stimulation of the vagus nerve increases bile bicarbonate secretion, whereas vagotomy decreases basal bile flow and bicarbonate output[60,61].

In early studies[62] it seemed that cholangiocytes might express both the M1 and the M3 acetylcholine (ACh) receptor subtypes and that the cholinergic agonist, carbachol, stimulates bile flow of the isolated perfused rat liver. These observations were not confirmed by successive investigations. In 1996, Nathanson *et al* demonstrated that, in accordance with the concept that cholangiocytes, but not hepatocytes, express ACh receptors, ACh did not affect the functions of hepatocytes, but elicited Ca^{2+} increase and oscillation in IBDUs, due to both an influx of extracellular Ca^{2+} and the mobilization of thapsigargin-sensitive Ca^{2+} stores[63]. A year later, Alvaro *et al* showed that cholangiocytes express, at the basolateral domain, M3 (but not M1 and M2) Ach receptors.[43] In that study it was shown that ACh has no effect on the basal activity of the Cl^-/HCO_3^- exchanger, but it significantly potentiates the stimulatory effect of secretin on this anion exchanger[43]. Presumably, the significance of ACh-regulation of bicarbonate secretion by bile ducts is to be researched in the need of bicarbonate-enriched secretion in the small intestine during the digestive phase, when, indeed, the parasympathetic system activity is high. By selectively interacting with M3 receptor subtypes, ACh induces a Ca^{2+}-calcineurin mediated potentiation of the secretin-induced adenylyl cyclase activity, which leads to activation of the Cl^-/HCO_3^- exchanger with bicarbonate secretion into bile[43]. Furthermore, recent studies have shown that ACh sustains cholangiocyte proliferation, since interruption of the cholinergic innervation by vagotomy induces a marked decrease in total bile duct mass caused by both impaired cholangiocyte proliferative capacity and intracellular cAMP levels, and enhanced cell death by apoptosis[64,65]. These studies also show that maintenance of intracellular cAMP levels (by chronic administration of forskolin) prevents the effects of vagotomy on cholangiocyte apoptosis, proliferation and secretion[65].

There is growing information regarding the role of adrenergic and dopaminergic innervation in the regulation of cholangiocyte proliferation and secretion. An intact sympathetic innervation is required for hepatocyte and cholangiocyte proliferation following partial hepatectomy[66]. Recent studies demonstrated that: (1) cholangiocytes from BDL rats express alpha-1, alpha-2, beta-1 and beta-2 adrenergic receptors; and (2) alpha-1 (but not beta-1) adrenergic receptor agonists increase secretin-stimulated ductal secretion[67]. Similar to what is shown in the gut, adrenergic innervation may play a role in counterbalancing the stimulatory effects of cholinergic

nerves[65] on ductal bile secretion in chronic cholestatic liver diseases.

In support of the concept that adrenergic innervation plays an important role in the regulation of cholangiocyte functions, administration of a single intraportal injection of 6-hydroxidopamine, which induces degeneration of dopaminergic terminal fibers[68,69], blunts the cholangiocyte functional and proliferative response to cholestasis and induces cell death by apoptosis[70]. Chronic administration of clenbuterol (a beta-2 adrenergic agonist)[71] and dobutamine (a beta-1 adrenergic agonist)[72] prevents the decrease in cAMP levels and secretion induced by 6-OHDA, maintains cholangiocyte proliferation and decreases cholangiocyte apoptosis due to 6-OHDA[70]. Furthermore, it has been shown that cholangiocytes express the D2 (but not the D1 and D3) dopaminergic receptors and that the D2 dopaminergic agonist, quinelorane inhibits secretin-induced ductal secretion in BDL rats through activation of the Ca^{2+}-dependent PKC gamma (but not PKC alpha, beta I and II) and inhibition of secretin-stimulated cAMP levels and PKA activity[73].

The relationship between the biliary epithelium and nerves appears to be more than what was thought at the beginning. Cholangiocytes express the receptor for neurotrophin Nerve Growth Factor (NGF), the activation of which strongly promotes biliary cell growth[74]. Moreover, it has been found that cholangiocytes themselves can produce and secrete NGF and that when this neurotrophin is immunoneutralized the growth of the biliary tree in the BDL rat is strongly diminished[74].

Altogether, these data suggest that the autonomic innervation plays a substantial role in the regulation of cholangiocyte biology, thus enlightening the need of studying whether the liver denervation after transplantation might affect the functions of the grafted biliary tree. Of major interest would also be to investigate whether other endogenous factors, instead of nerves, can support cholangiocyte functions in the denervated organ. In this view, some evidence suggest that bile acids could be important. It has been found that administration of taurocholic[64] or ursodeoxycholic acid[75] counteracts the loss of bile ducts induced by cholinergic denervation in the BDL rat. Similarly, taurocholic acid administration also prevents the loss of bile ducts induced by adrenergic denervation[76].

NERVOUS AND NEUROENDOCRINE REGULATION OF BILIARY CARCINOGENESIS

Cholangiocarcinoma, the primary cancer that originates from the epithelial cells lining the bile ducts, is a devastating malignancy characterized by poor prognosis and high mortality[6,77]. The only curative treatment, even if only possible at an early stage of this neoplasm, is surgical. However, patients often present this tumor at an advanced stage, when a curative surgery is unlikely. In fact, at the time of diagnosis, more than two-thirds of patients affected by biliary tract cancer present as an unresectable disease[78] and patients with an operable tumor have a high rate of recurrence. Overall survival rate, including resected patients is quite poor, with less than 5% of patients surviving 5 years, a rate which has remained unchanged over the past 30 years[77]. Chemotherapy and radiation therapy have been used in an attempt to control this disease and improve survival and quality of life of patients with unresectable, recurrent and metastatic cholangiocarcinoma, but these therapies have not shown to be effective in prolonging long-term survival. For these reasons there is a compelling need to discover novel molecules and agents to target the cholangiocarcinoma cells and to regulate their destructive growth[79]. There is growing information regarding the role of nerves and neuropeptides as modulators of cholangiocyte function and metabolism[14]. Moreover, several reports suggest that nervous stimuli are involved in the regulation of growth of biliary malignancies[16,17,80].

Table 3 Neuroendocrine hormones affecting cholangiocarcinoma cell biology

Hormone	Receptor	Effect on cholangiocarcinoma cell biology	Reference
Estrogens	ER	Stimulated SK-ChA-1 human cholangiocarcinoma cell growth	81
		Tamoxifen induced dose-dependent growth inhibition of OZ and SK-ChA-1 human cells *in vitro*; reduced growth of a SK-ChA-1 tumor cell xenografts implanted in athymic nude mice	81
		Tamoxifen stimulated SK-ChA-1 human cholangiocarcinoma apoptotic cell death	82
		Tamoxifen induced human cholangiocarcinoma cell apoptosis *in vitro* and inhibited tumor xenograft growth after pretreatment with IFN-gamma	83, 84
Gastrin	CCK-B /gastrin	Inhibited Mz-ChA-1, HuH-28, and TFK-1 human cell lines proliferation and induced Mz-ChA-1 cell apoptosis	90
CCK	CCK	Reduced the growth of SLU-132 human tumor xenografts implanted in nude mice; stimulated the release of carcinoembryonic antigen (CEA) by SLU-132 cells	93

Regulation of malignant cholangiocyte growth by neuroendocrine hormones (Table 3,Table 4)

The involvement of neuroendocrine system in regulating cholangiocarcinoma cell proliferation has been suggested by several studies. Tamoxifen, an estrogen antagonist, cause a dose-dependent decrease of proliferation of two human cholangiocarcinoma cell lines *in vitro*[81]. On the other hand, 17-β estradiol stimulate human cholangiocarcinoma cell growth *in vitro*[81]. In addition, tamoxifen induced a growth reduction of a cholangiocarcinoma tumor implanted in athymic nude mice[81]. Tamoxifen exerts its inhibitory effect in human cholangiocarcinoma cells by stimulating apoptotic cell death and this is likely mediated through the Fas/APO-1 (CD95) signaling pathway via a calmodulin-dependent mechanism[82].

A further study showed that tamoxifen exposure to human cholangiocarcinoma after pre-treatment with

IFN-gamma allows for induction of apoptosis *in vitro* and significant inhibition of tumor growth[83]. Thus, the combination of IFN-gamma and estrogen antagonists, including tamoxifen, could be useful to have a sustained and valid inhibitory effect on cholangiocarcinoma cell growth[83,84].

Gastrointestinal polypeptide hormones regulate the growth of various normal gastrointestinal tissues as well as certain visceral cancers. Gastrin, which belongs to the superfamily of cholecystokinin receptors (CCK-A, CCK-B/gastrin receptors), is a trophic factor within the normal gastrointestinal tract and is also a mitogen for a number of gastrointestinal and non-gastrointestinal tumors such as gastric, colonic and pancreatic[85-88]. In contrast, CCK-B/gastrin receptor signaling in the human pancreatic cell lines MiaPaca-2 and Panc-1 leads to inhibition of cell growth[89]. It has been shown that malignant cholangiocytes express gastrin receptors[90] and that gastrin, interacting with CCK-B/gastrin receptors, inhibited the proliferation of Mz-ChA-1, HuH-28 and TFK-1 cholangiocarcinoma cell lines, also inducing apoptosis in Mz-ChA-1 cells by activation of the Ca^{2+}-dependent PKC-α signaling[90]. The blockage of CCK-B receptors did not totally reverse the inhibitory effect of gastrin on Mz-ChA-1 cell growth. This finding could be explained by the fact the gastrin may exert its effect not only through CCK-B/gastrin receptors, but also through other receptor types, as suggested by studies in other carcinomas cell lines (*e.g.* colonic cancer cells)[91]. This partial inhibitory effect by CCK-B/gastrin receptor inhibitors might also be due to altered processing of the CCK/B receptor by malignant transformed cells, as previously described[92]. A previous study by Hudd *et al* showed that human cholangiocarcinoma cells expressed CCK receptors and chronic treatment with CCK octapeptide reduced the growth of human cholangiocarcinoma tumors implanted in nude mice[93].

Somatostatin receptors (SS), most commonly the SS receptor type 2 (SSTR2), have been described in several neuroendocrine and epithelial malignancies[94], such as in malignant cholangiocytes[95,96]. Studies demonstrated that somatostatin and its analogues inhibit *in vitro* cholangiocarcinoma cell proliferation. Indeed, chronic administration of lanreotide, a long-acting SS analogue, reduces the growth of human cholangiocarcinoma cells when implanted in athymic mice[95,96] and the inhibitory effect of somatostatin in cholangiocarcinoma cell growth was accompanied by no changes in cellular cyclic adenosine monophosphate (cAMP) or calcium intracellular levels. Furthermore, Zhao *et al* showed that octreotide inhibits cholangiocarcinoma cells growth through G0/G1 cell cycle arrest rather than through the process of apoptosis. These effects were partially mediated by enhancing the expression of $p27^{kip1}$, and by decreasing the amounts of cyclin E-CDK2 complex[95]. Taken together, these findings suggested possible use of SS analogues in the diagnosis or therapy of cholangiocarcinoma. However, a subsequent phase II study showed absence of therapeutic efficacy of the somatostatin analogue lanreotide in the treatment of advanced primary hepatic cholangiocellular cancer and gallbladder adenocarcinoma, despite *in vivo* somatostatin-receptor expression[97].

Table 4 Neuroendocrine hormones affecting cholangiocarcinoma cell biology

Hormone	Receptor	Effect on cholangiocarcinoma cell biology	Reference
Somatostatin, analogues (octreotide, lanreotide)	$SSTR_2$	Inhibited human cholangiocarcinoma cell proliferation *in vitro* and reduced human cholangiocarcinoma cell growth implanted in athymic mice	96
		Inhibited RBE, NEC, QBC939, and SSP-25 cell proliferation *in vitro* through cell cycle arrest and QBC939 xenografts growth	95
GABA	$GABA_{A,-B,-C}$	Inhibited human Mz-ChA-1, HuH-28 and TFK-1 cell proliferation *in vitro*; reduced malignant cholangiocyte migration. Reduced Mz-ChA-1 xenograft tumor growth implanted in athymic mice	98
NPY	NPY-Y5	Inhibited Mz-ChA-1 cell proliferation *in vitro*	99

Liver represents the most important site of GABA synthesis and metabolism outside the central nervous system. Recently, our group demonstrated that cholangiocarcinoma cells express $GABA_{A,-B,-C}$ recep-tors and respond to GABA stimulation with growth inhibition. GABA inhibitory effect on malignant cholangiocyte proliferation was evident *in vitro* and also *in vivo*, by reducing the growth of cholangiocarcinoma tumors injected subcutaneously in nude mice. Moreover, GABA has been shown to be able to inhibit malignant cholangiocyte migration, a peculiar characteristic of the cholangiocarcinoma cells[98].

Another neurotransmitter available in the liver parenchyma and biliary tract is the neuropeptide Y (NPY)[99]. Recent preliminary data from our group showed that NPY inhibits cholangiocarcinoma growth by interaction with a G-protein coupled receptor by Ca^{2+} dependent modulation of Src/ERK1/2 phosphorylation[99].

Regulation of malignant cholangiocyte growth by neurotransmitters (Table 5)

Autonomic nervous system regulates the growth of several tumors[100-102]. For example, alpha-blockers, terazosin and doxazosin, suppress prostate growth by inducing apoptosis among the epithelial cells in the benign and malignant prostate[103]. Also, phenylephrine reduced $HepG_2$ cell growth through $alpha_{1B}$-adrenergic receptors activation[104]. Moreover, activation of a β-2 adrenergic receptor/Gsα fusion protein leads to the inhibition of cAMP-sensitive S49 lymphoma and carcinoma carB cells proliferation *in vitro*[105].

A specific role of sympathetic nervous system in the regulation of cholangiocarcinoma growth has been described by Kanno *et al*, that showed that cholangiocarcinoma cell lines Mz-ChA-1 and TFK-1 express the α_{2A}-, α_{2B}-, α_{2C}- adrenergic receptor subtypes[80]. Furthermore, stimulation of malignant cholangiocytes by UK14, 304, an α_2-adrenoreceptor agonist, causes up-regulation of cAMP, which inhibits EGF-induced MAPK activity through

Table 5 Neurotransmitters/neurotrophins affecting cholangiocarcinoma cell biology

Peptide	Receptor	Effect on cholangiocarcinoma cell biology	Reference
Epinephrine/ Norephinephrine	α_2	UK 14,304 inhibited human Mz-ChA-1 and TFK-1 cell proliferation *in vitro*	[80]
Acetylcholine	M1	Carbachol produced an increase of IP3 and Ca^{2+} intracellular levels in Mz-ChA-1 cells	[62]

an acute increase of Raf-1 and sustained activation of B-Raf[80], thus reducing tumor cell proliferation. Since inhibition of cholangiocarcinoma cell growth through activation of α_2-adrenergic receptor occurred downstream to Ras, this study suggested that adrenergic stimulation or other stimulants of cAMP may overcome the Ras mutations present in malignant cholangiocytes and offer a new therapeutic approach in patients with cholangiocarcinoma[80].

Other studies showed that Mz-ChA-1 cells express muscarinic acetylcholine (ACh) receptors[62]. In support of the hypothesis that such receptors modulate cholangiocarcinoma cell growth, stimulation of malignant biliary cells with carbachol, a muscarinic acetylcholine receptor agonist, triggers IP_3 formation and a subsequent increase of intracellular Ca^{2+} levels[62]. Even if several studies show IP_3 and Ca^{2+} levels play an important role in the inhibition of cholangiocarcinoma cell growth after stimulation with several molecules[13,90,98]. the specific action of parasympathetic nervous system in modulating cholangiocarcinoma cell growth is still unknown.

However, recent data showed that muscarinic receptors are directly activated by other molecules. In fact, recent studies showed that bile acids, interacting with M3 Ach receptors, transactivate EGFR and stimulate colon cancer cell proliferation by inducing p90RSK phosphorylation via a calcium-, MEK- and MAPK-dependent pathways[106].

Recent findings suggest that cholangiocarcinoma and other liver tumors could arise from activated hepatic progenitor cells[107-109]. Several evidences indicate that hepatic progenitor cell activation in diseased liver is regulated by neural and neuroendocrine factors such as vagal innervation[110,111], suggesting an important involvement of autonomic nervous system in developing biliary carcinogenesis.

SUMMARY AND FUTURE PERSPECTIVES

The amount of evidences indicating the substantial role of neuroendeocrine hormones, neuropeptides and neurotransmitters in modulating malignant and non malignant cholangiocyte biology is rapidly increasing. There is still much to understand on how actually these peptides create a network that affects the response of biliary cells to liver injury. On the other hand the other frontier is no doubt represented by the identification of the most proper way of modulating such a network of peptides. Will we be able to interfere with it in order to divert or relent the progression of cholangiopathies and cholangiocarcinoma?

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