

Review

Genetic and Molecular Abnormalities in Cholangiocarcinogenesis

VICTOR J. HASSID¹, FRANK A. ORLANDO², ZIAD T. AWAD¹, DONGFENG TAN³, THAER KHOURY⁴, BESTOUN H. AHMED¹ and SADIR J. ALRAWI⁵

¹Department of Surgery University of Florida, Jacksonville, FL;

²Department of Surgery, University of Florida, Gainesville, FL;

³Department of Pathology, University of Texas M.D. Anderson Cancer Center, Houston, TX;

⁴Department of Pathology, Roswell Park Cancer Institute, Buffalo, NY;

⁵Surgical Oncology Specialties, Cancer Center of Jacksonville, FL, U.S.A.

Abstract. Cholangiocarcinomas are biliary tree neoplasms of cholangiocyte origin. Several clinical risk factors are associated with cholangiocarcinogenesis. During the last decade, there has been an increasing interest in the causative molecular mechanisms of cholangiocarcinoma because of its poor prognosis and the lack of effective therapies. A better understanding of cholangiocarcinoma tumor initiation, promotion, and progression, as well as neurotransmitter, neuroendocrine, and endocrine growth effects, may elucidate molecular targets for diagnostic and therapeutic purposes.

Cholangiocarcinomas are biliary tree neoplasms that arise from bile duct epithelial cells known as cholangiocytes. Microscopically, their most common feature is a well- to moderately differentiated adenocarcinoma. Cholangiocarcinomas usually occur at biliary duct confluences (Klatskin tumors), but they can also present within the liver or distal to the hilum. Although the incidence of cholangiocarcinoma is low, with approximately 8 cases per million per year in the United States, it is increasing globally (1-3).

Several important clinical risk factors exist for cholangiocarcinoma such as female gender, Caroli's disease, congenital choledochal cysts, primary sclerosing cholangitis, hepatolithiasis, ampulla of Vater adenomas, *Opisthorchis viverrini* and *Clonorchis sinensis* liver fluke infestation,

Salmonella typhi infection, and obesity. Gallbladder diseases associated with cholangiocarcinoma include symptomatic cholelithiasis, polyps greater than 1 cm, and porcelain gallbladder. Several of the above risk factors play a role in biliary obstruction, chronic inflammation, and consequential cholangiocyte injury, which are well established in cholangiocarcinoma development. Hepatocytes, sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells in the biliary microenvironment secrete inflammatory cytokines, and it is these cytokines that may induce malignant transformation in cholangiocytes (4, 5).

Current molecular mechanisms of cholangiocarcinogenesis focus on growth regulatory genes and chronic biliary inflammation. Although several studies have clarified the link between chronic cholestasis and endogenous neuroendocrine peptides in the acquisition of a malignant phenotype, a more complete understanding of the genetic profile of cholangiocarcinoma is still needed to develop potentially effective, targeted molecular therapy (6, 7).

Operative intervention is currently the only "curative" treatment for early-stage cholangiocarcinoma; however, the recurrence rate is high. Unfortunately, tumors are usually diagnosed at an advanced stage when the chance of curative resection is very limited (4). Mortality is high and the 5-year survival is less than 5% (8). Chemotherapy and radiation have not yet been proven to prolong long-term survival (9).

Tumor Initiation

Genetic and molecular abnormalities contribute to cholangiocarcinoma tumor initiation, promotion and progression (Figure 1). A fundamental step in carcinogenesis is the development of autonomous proliferative signaling. A malignant cell phenotype is initiated when mutant

Correspondence to: Sadir J. Alrawi, MD, Surgical Oncology Specialties, Cancer Center of Jacksonville, 7751 Bay Meadows Rd. E, Jacksonville, FL 32256, Tel: +190 46455045, Mobile: +190 46251532, Fax: +190 46455856, e-mail: alrawi3gad@aol.com

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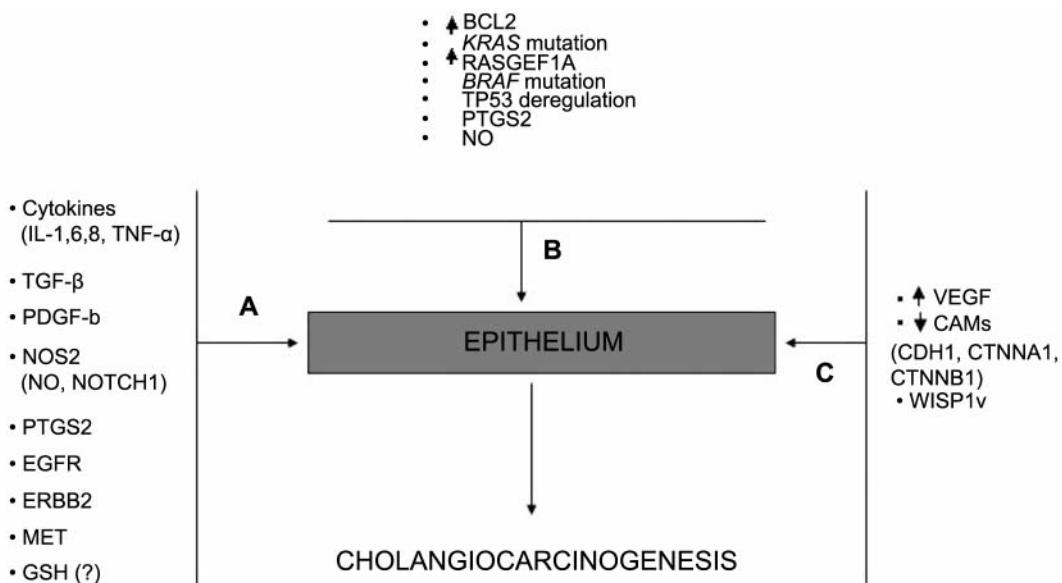


Figure 1. Molecular basis of cholangiocarcinogenesis. A: Tumor initiation; B: tumor promotion; C: tumor progression.

cholangiocytes produce mitogens that activate local cellular receptors and intracellular signaling pathways (4, 6). Cholangiocytes secrete cytokines such as IL(Interleukin)6, transforming growth factor-beta (TGF-beta), IL8, tumor necrosis factor-alpha (TNF-alpha), and platelet-derived growth factor (PDGF) beta chain, all of which regulate biliary cell homeostasis through paracrine signaling (10, 11). During carcinogenesis, aberrant cytokine stimuli alter cholangiocyte intracellular signaling, which contributes to the development and growth of biliary tract carcinomas (6, 12).

Cholangiocyte cytokines stimulate inducible nitric oxide synthase (NOS2) to produce nitric oxide (NO), a known DNA mutagen linked to malignant transformation (5, 13). The generation of NO is also important for bile duct development because it induces *Notch1* expression (14, 15). The four *Notch* genes identified in mammals (*Notch 1-4*) are expressed in a wide variety of cells and play a significant role in cellular differentiation. The activation of Notch by cell-to-cell interaction causes a transcriptional silencing effect that inhibits differentiation in some cells but not in others (16-18). While the Notch pathway is known to be associated with pancreatic carcinogenesis in rats and humans (19), this same pathway may also have a role in cholangiocarcinogenesis via NOS2.

Cyclooxygenase-2 (prostaglandin-endoperoxide synthase 2, PTGS2) is also implicated in the initiation of malignant cholangiocytes (20). PTGS2 is up-regulated in murine and rat models of biliary adenocarcinoma, while the antisense depletion of PTGS2 has been observed to inhibit tumor cell

proliferation (21, 22). Oxysterols are the oxidative derivatives of the bile cholesterol present during cholestasis and are also associated with biliary carcinogenesis. Human cholangiocarcinoma cell lines exposed to oxysterols *in vitro* have elevated PTGS2 expression (23, 24), further supporting the association between inflammation and cholangiocarcinoma.

The oncogenes *ERBB2* and *MET* have also been shown to increase PTGS2 expression, and both are involved in cholangiocyte carcinogenesis (9, 25). Cholangiocarcinoma cell lines strongly overexpress *ERBB2*, and *MET* expression is increased in the early phases of cholangiocarcinogenesis (25-27). Normal rat cholangiocytes transfected with *Erbb2* underwent malignant transformation with molecular features resembling human cholangiocarcinoma (28). In addition, the *MET* receptor is bound by hepatocyte growth factor (HGF), and HGF overexpression in cholangiocarcinoma has been shown to have a mitogenic effect on cholangiocytes (29).

The epidermal growth factor receptor (EGFR) is activated by bile acids and has been linked to cholangiocarcinoma growth. The bile acid-dependent activation of EGFR requires metalloproteinase activity and functions with phosphoinositide 3-kinase (PIK3CA) signaling to promote the expression of anti-apoptotic molecules (30). Survival and proliferative signaling are therefore stimulated by EGFR activation through PIK3CA. Furthermore, EGFR expression is prognostic and an indicator of intrahepatic cholangiocarcinoma recurrence (31).

The acute phase proteins IL6 and TGFB1 affect the growth of biliary epithelial cells (12). IL6 secretion increases during the course of chronic inflammation and biliary duct neoplasia, resulting in sustained proliferation by an autocrine/paracrine

mechanism (32). TGFB1 regulates cellular proliferation, differentiation, migration, and apoptosis, thereby acting as a cholangiocyte tumor suppressor (12, 33, 34). However, mutations in *TGFB1* (TGF beta receptor 1) and *SMAD4* (alias *DPC4*) alter TGFB1 signaling in cholangiocarcinoma cells, allowing them to escape from TGFB1 tumor suppression (12, 34, 35). SMAD4 is an important component of the TGFB1 pathway, and mutations causing loss of its expression have been described in biliary malignancies, particularly extrahepatic cholangiocarcinoma (36, 37). *SMAD4* and *PTEN* are tumor suppressor genes that function synergistically in cholangiocarcinogenesis, and their disruption in a mouse model resulted in the development of biliary malignancies (38).

The main intracellular defense against oxidative stress during inflammation is reduced glutathione (GSH). GSH maintains proteins and other molecules in the reduced state and participates in the detoxification of many molecules (39). A GSH deficiency can lead to apoptosis deregulation and DNA damage (40). Although the role of GSH in the cholangiocarcinogenic process is not completely understood, reduced GSH levels have been found in cells with chronic biliary diseases and in experimentally induced cholestasis (39).

Tumor Promotion

Apoptosis is the mechanism of programmed cell death allowing organisms to delete cells that are unable to repair DNA damage (41). Abnormalities of this mechanism promote tumorigenesis because mutated cholangiocytes may subsequently result in malignancy (41). The inhibition of apoptosis in cholangiocarcinoma has been linked to increased *BCL2* expression, *KRAS* mutation, and/or *TP53* deregulation (40). The anti-apoptotic protein *BCL2* is expressed by bile ductules and inhibits cytochrome c release from mitochondria, thereby preventing caspase-3 activation (42, 43). Point mutations of the *KRAS* proto-oncogene are frequently present in cholangiocarcinoma specimens arising near the hepatic hilum, especially when there is lymph node metastasis (44, 45). Mutations of the tumor suppressor *TP53* have also been described in intrahepatic cholangiocarcinomas (6).

In addition to initiating tumor formation *via* mutagenesis, NO inhibits apoptosis in human cholangiocarcinoma cell lines through the nitrosylation of caspase 9 (5, 46). Interestingly, NOS2 also promotes mouse cholangiocyte growth by up-regulating PTGS2 (47). Administration of the selective PTGS2 inhibitor celecoxib enhances apoptosis in rat cholangiocarcinoma cells (22), suggesting that PTGS2 deregulation may promote carcinogenesis.

BRAF is a RAF family gene activated by KRAS and frequently mutated in cholangiocarcinomas (48). Mutations of either *KRAS* or *BRAF* are frequently encountered in cholangiocarcinogenesis (48). *RASGEF1A* (RasGEF domain

family, member 1A) is a novel gene encoding a guanine nucleotide exchange factor for RAS-like small GTPases that has elevated expression in the majority of human intrahepatic cholangiocarcinomas (49). The suppression of *RASGEF1A* expression by interfering RNA (RNAi) reduces the growth rate of human cholangiocarcinoma cells, demonstrating the potential of *RASGEF1A* as a therapeutic target in intrahepatic cholangiocarcinoma (49).

Tumor Progression

During tumor progression, neovascularization (*de novo* formation of functional microvascular networks) and angiogenesis (pre-existing capillary extension) deliver nutrients and oxygen to malignant cells and help prevent the tumor mass from outgrowing the native vascular network. Vascular endothelial growth factor (VEGF) is an important signaling protein for both neovascularization and angiogenesis in cholangiocarcinoma progression (4, 6). Although TGFB1 is a known tumor suppressor, it is coexpressed with VEGF in human cholangiocarcinoma tumors and has been implicated as an angiogenesis activator in an *in vitro* model (50).

Metastasis is another sign of tumor progression, and VEGF overexpression in intrahepatic cholangiocarcinoma is associated with liver metastasis (31). The highly invasive and metastatic behavior of cholangiocarcinoma is also linked to the expression of matrix metalloproteinases, such as human aspartyl b-hydroxylase, and proteins related to the connective tissue growth factor (CTGF) family (51, 52). WISP1 is a member of the CTGF family encoding the WNT1 inducible signaling pathway (WISP) protein 1. The expression of the splicing variant WISP1v is associated with perineural and lymphatic tumor invasion and is therefore a poor prognosticator in cholangiocarcinoma (53, 54).

Altered cell adhesion molecule (CAM) expression is an additional factor contributing to tumor progression. Down-regulation of CDH1 (E-cadherin), CTNNNA1 (alpha-catenin) and CTNNB1 (beta-catenin) is associated with high-grade cholangiocarcinomas but not with vascular invasion or metastatic behavior (55). Nevertheless, the interaction of CTNNB1 with MUC1 and MET has been shown to enhance the invasive and metastatic properties of cholangiocarcinoma (56).

Neurotransmitter, Neuroendocrine and Endocrine Growth Effects

Certain cholangiocarcinoma cell lines express several alpha-adrenergic receptor subtypes, and stimulation of the alpha2-adrenoreceptors *in vitro* up-regulates cAMP, inhibits EGF-induced MAPK1 activity, and reduces cell proliferation (57). Muscarinic acetylcholine receptors are located on the surface of the same cell lines; however, the effect of the

parasympathetic nervous system on cholangiocarcinoma growth is still unknown (58). Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter synthesized and metabolized in the central nervous system and liver. GABA inhibits both cholangiocarcinoma cell migration *in vitro* and xenograft tumor growth in mice via GABA(A), GABA(B), and GABA(C) receptors on cholangiocytes (59).

Gastrin, cholecystokinin octapeptide (CCK8), and the longer-acting somatostatin analogs octreotide and lanreotide are all neuroendocrine peptides known to regulate cholangiocarcinoma growth (7). The activation of gastrin receptors expressed by cholangiocarcinoma cells leads to inhibition of proliferation and acceleration of apoptosis through a Ca²⁺-dependent signaling pathway (60). However, gastrin's inhibitory or stimulatory effect on cancer cell growth is specific to the predominant intracellular isoform of cAMP-dependent protein kinase A (61).

Several therapeutic models have demonstrated the efficacy of neuropeptide drugs to alter malignant growth. Chronic CCK8 treatment reduced the growth of human cholangiocarcinoma xenografts in nude mice (62). In cholangiocarcinoma models expressing the somatostatin receptor 2, octreotide attenuates *in vitro* cholangiocyte proliferation and lanreotide inhibits *in vivo* tumor growth of human cholangiocarcinoma xenografts (63, 64).

The endocrine system influences cholangiocarcinogenesis via the estrogen receptor (ESR1), a ligand-activated transcription factor with multiple domains for hormone binding, DNA binding, and transcription activation. The ESR1 agonist 17-beta-estradiol stimulates cholangiocarcinoma cell growth *in vitro*, and the ESR1 antagonist tamoxifen inhibits the growth of human cholangiocarcinoma cells both *in vitro* and *in vivo* (65). Moreover, tamoxifen administered to human cholangiocarcinoma cells following pre-treatment with interferon-gamma induces *in vitro* apoptosis and inhibits tumor growth in mouse xenografts (66, 67). Further *in vivo* studies are required to clearly define the association between estrogens and cholangiocarcinoma.

Conclusion

Continued genetic and molecular research is crucial because of the rising incidence of cholangiocarcinoma and the lack of effective treatments. The high frequency of late-stage diagnosis is a major difficulty facing surgeons treating cholangiocarcinoma because this limits the possibility of a curative resection. The development of novel therapeutic approaches based on tumor biology is among the goals of modern medicine. Putative molecular targets such as PTGS2 and NOS2 inhibitors can potentially affect the incidence and growth of cholangiocarcinoma when used as prophylactic and therapeutic options, respectively. Future studies focusing on the discussed

genetic and molecular targets will help develop more effective therapies to treat cholangiocarcinoma when curative resection is unlikely.

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