Recent advances in the regulation of cholangiocarcinoma growth

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1Department of Internal Medicine, Texas A&M Health Science Center College of Medicine; 2Digestive Disease Research Center and 3Department of Research and Education, Scott & White Hospital; and 4Division of Research, Central Texas Veterans Health Care System, Temple, Texas
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Francis H, Alpini G, DeMorrow S. Recent advances in the regulation of cholangiocarcinoma growth. Am J Physiol Gastrointest Liver Physiol 299: G1–G9, 2010. First published April 29, 2010; doi:10.1152/ajpgi.00114.2010.—Cholangiocarcinomas arise after the neoplastic transformation of the cholangiocytes that line the intra- and extrahepatic biliary epithelium. Symptoms usually do not present until late in the course of the disease, at which time they are relatively difficult to treat and display a poor prognosis. Because of the relative rarity of this disease, the overall volume of research into the molecular pathophysiology associated with this disease is small compared with other more prevalent tumors. However, the incidence of this devastating cancer is on the rise and renewed efforts to understand the pathogenesis of cholangiocarcinoma is needed to design novel therapeutic strategies to combat this disease. This review summarizes the recent advances into our knowledge and understanding of cholangiocarcinoma and highlights potential novel therapeutic strategies that may prove useful to treat this deadly disease.

General Background

Cholangiocarcinoma arises from the neoplastic transformation of cholangiocytes and can be either intrahepatic, perihilar, or distal extrahepatic tumors (3). Typically, cholangiocarcinomas are adenocarcinomas and have a poor prognosis and limited treatment options. This is due at least in part to the late presentation of symptoms and the relative resistance to current treatment options (81).

The incidence of both intra- and extrahepatic cholangiocarcinoma is typically more prevalent in Asian countries (74). The mortality rates for intrahepatic cholangiocarcinoma have increased since the 1970s, whereas deaths from extrahepatic cholangiocarcinoma have declined in most countries (74). There is a slight preponderance for cholangiocarcinoma in men (93), and the incidence in both sexes increases with age (74).

Risk Factors

Cholangiocarcinoma occurs with a varying frequency in different regions of the world. This can be explained in part by the distribution of risk factors in geographic regions and ethnic groups (7). The common link between these regional risk factors seems to involve chronic inflammation and biliary irritation (38).

The prevalence of cholangiocarcinoma in Asian countries shares a relationship with infections such as liver flukes, hepatitis B, and hepatitis C (7). In contrast, ~90% of patients diagnosed with cholangiocarcinoma in Western countries do not have any recognized risk factors (7). However, the remaining 10% of cases are associated with certain risk factors. Apart from factors related to chronic inflammation, both intra- and extrahepatic cholangiocarcinomas are well-known complications of primary sclerosing cholangitis (19). Other known risk factors include obesity, hepatolithiasis, bacterial infection, and/or bile stasis-related chronic cholangitis (14, 15, 19).

Epigenetic Changes

Neoplastic transformation of normal cells into their malignant counterparts often requires a series of genetic changes. These changes can range from “simple” mutations in the genes themselves, that ultimately lead to loss-of-function or gain-of-function changes in key genes that are responsible for the control of apoptosis and cell cycle progression respectively, to more complex changes in nonprotein factors (e.g., RNA and DNA) that regulate the control of specific gene expression (18, 48, 49). These more complex changes can be classified as either genetic or epigenetic changes (18, 48, 49). Recent data suggest that both genetic and epigenetic changes are required for transformation, promotion, and progression of cholangiocarcinoma (58–60, 88, 90, 91, 102).

Hypermethylation. A large number of genes are regulated by hypermethylation in cholangiocarcinoma. The functions of these genes range from tumor suppressors, cell cycle inhibitors, DNA repair, as well as regulators of cell adhesion and invasion (84). The most well-characterized epigenetic change with respect to cell cycle inhibitors is the pl16INK4a gene, which has been described in up to 83% of cholangiocarcinomas (88, 102). This gene is responsible for binding to the cyclin-dependent kinase 4 (CDK4) and inhibits its ability to interact with cyclin D1 (63). In the absence of pl16INK4a activity, such as after
promoter methylation, CDK4 binds to cyclin D1, which subsequently leads to unchecked entry into the S phase of the cell cycle (63). There also appears to be increased incidence of hypermethylation of the related p14ARF occurring in 25% of cholangiocarcinoma samples studied (91). Many other cell cycle entry inhibitors have been shown to be hypermethylated, including p16INK4a (50% of tumors studied) (102) and 14-3-3 sigma (59.5% of tumors studied) (54).

In addition, the expression of many tumor suppressor genes are repressed in cholangiocarcinomas (90). The most striking of these is Semaphorin3B, which was found to be methylated in 100% of the cholangiocarcinoma cases studied (90). RassF1A (102) and p73 (102) are also hypermethylated and suppressed in 65.3 and 36.1% of cholangiocarcinoma cases studied, respectively.

Several DNA repair genes have been shown to be hypermethylated in cholangiocarcinoma. Hypermethylation of the hMLH1 mismatch repair gene promoter has been shown to occur in up to 23.6% of cholangiocarcinomas (102), which has previously been revealed to lead to microsatellite instability in other tumor types (31). Another gene, O6-alkylguanine-DNA methyltransferase (MGMT), is silenced in up to 33% of cholangiocarcinoma tumors studied (102). This gene is an important suicide enzyme involved in the defense against O6-alkylating endogenous metabolites and environmental carcinogens. Interestingly, transcriptional repression of MGMT was associated with the accumulation of GC-AT transitional mutations in the p53 gene and, to a lesser extent, the k-ras gene in cholangiocarcinoma (51, 102). Lastly, the glutathione S-transferase P1 (GSTM1) gene, which inactivates electrophilic carcinogens by conjugation with glutathione, is hypermethylated in cholangiocarcinoma, occurring in 6–34% of cases studied (54, 102).

Lastly, hypermethylation of E-cadherin, a calcium-dependent cell adhesion molecule that suppresses metastatic processes and tumor cell invasion (25, 69, 82), has been demonstrated in up to 48% of cholangiocarcinoma samples studied (50, 54, 91, 102). Downregulation of this gene by epigenetic changes has been reported in other cancers (25), and that reexpression can be induced by treatment with a demethylating agent in cholangiocarcinoma (26).

A summary of the incidence of the above-mentioned hypermethylation event is found in Table 1.

**Table 1. Summary of genes that are silenced by hypermethylation in CCA**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Function</th>
<th>Incidence in CCA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3 sigma</td>
<td>Cell cycle regulator</td>
<td>59.5%</td>
<td>54</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Cell adhesion</td>
<td>43%</td>
<td>50, 54, 91, 102</td>
</tr>
<tr>
<td>EGFR</td>
<td>Growth Factor</td>
<td>ND</td>
<td>96</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Inactivation of carcinogens</td>
<td>6–34%</td>
<td>54, 102</td>
</tr>
<tr>
<td>hMLH1</td>
<td>DNA mismatch repair</td>
<td>25%</td>
<td>102</td>
</tr>
<tr>
<td>MGMT</td>
<td>Methyl transferase</td>
<td>11–33%</td>
<td>54, 102</td>
</tr>
<tr>
<td>p14ARF</td>
<td>Cell cycle regulator</td>
<td>38%</td>
<td>54, 102</td>
</tr>
<tr>
<td>RassF1A</td>
<td>Cell cycle regulator</td>
<td>12–50%</td>
<td>50, 102</td>
</tr>
<tr>
<td>RassF1A</td>
<td>Tumor suppressor</td>
<td>36%</td>
<td>102</td>
</tr>
<tr>
<td>SOCS-3</td>
<td>Inhibits inflammation</td>
<td>26–65%</td>
<td>50, 102</td>
</tr>
<tr>
<td>Semaphorin3B</td>
<td>Tumor suppressor</td>
<td>100%</td>
<td>90</td>
</tr>
<tr>
<td>SOCS-3</td>
<td></td>
<td></td>
<td>46</td>
</tr>
</tbody>
</table>

CCA, cholangiocarcinoma; ND, not determined.

**Association with Inflammation**

Chronic inflammation is a recognized risk factor for the development of cholangiocarcinoma (3, 81). Indeed, sustained overexpression of the cytokine interleukin-6 (IL-6) plays an integral role in cholangiocarcinoma biology (85, 103). This aberrant overexpression of IL-6 is a consequence of the epigenetic silencing of the suppressor of cytokine signaling 3 (SOCS-3) (46). SOCS-3 promoter methylation was observed in a subset of cholangiocarcinoma samples and cholangiocarcinoma cell lines (46). Forced overexpression of SOCS-3 in these cell lines effectively reduced the IL-6-mediated signal transduction cascade (46), therefore the loss of this negative regulator of IL-6 in cholangiocarcinoma may contribute to increased expression and activity of inflammatory molecules seen in cholangiocarcinoma.

A downstream consequence of aberrant IL-6 expression may be the further hypermethylation of the promoter regions of a number of critical target genes in cholangiocarcinoma. IL-6 has been shown to regulate the enzyme activity of one of the DNA methyltransferases responsible for the hypermethylation of promoter regions (44). In cholangiocarcinoma cells, IL-6 overexpression resulted in the altered promoter methylation of a number of genes including the epidermal growth factor receptor (EGFR) (96). EGFR promoter methylation was de-
Increased and gene and protein expression were increased by IL-6 (96), suggesting that the epigenetic regulation of gene expression by the inflated IL-6 expression seen in cholangiocarcinoma can contribute to tumor progression by altering the expression of growth regulatory pathways, such as those involving EGFR, caspase 8, and survivin in a manner that promotes survival and growth of the tumor cell (96).

Role of Growth Factors in Cholangiocarcinoma Growth

Immunohistochemical studies have shown (100) that the overexpression of NGF-β and VEGF-C occurred in ~57.1 and 46.4% of cholangiocarcinoma samples, respectively. A number of human cholangiocarcinoma cell lines and samples express VEGF-A and VEGF receptors (VEGFRs) and the angiogenic factors angiopoietin-1, -2, and thrombospondin-1 (4, 86). Also, estrogens modulate cholangiocarcinoma growth. For example, tamoxifen (estrogen receptor antagonist) inhibits cholangiocarcinoma growth through calmodulin targeting of protein kinase B and cellular-FLICE inhibitory protein (75). An interaction between VEGF and estrogens in the regulation of cholangiocarcinoma growth has been defined in vivo and in vitro (55). Specifically, estrogens have been shown to stimulate the growth of human cholangiocarcinoma by inducing the expression and secretion of VEGF (55).

Other studies have shown that intrahepatic and extrahepatic cholangiocarcinoma samples overexpress EGFR and VEGF (42, 104). The studies have also shown that 1) EGFR expression is associated with cholangiocarcinoma progression and 2) VEGF expression regulates metastasis in cholangiocarcinoma (104). In support of this notion, inhibition of VEGFR and EGFR signaling with vandetanib (ZD6474, a tyrosine kinase receptor. Gastrin inhibits the growth of cholangiocarcinoma cells lines by Ca2+-dependent protein kinase C-α activation of apoptosis (47). Cholecystokinin increases the synthesis and release of carcinomaembryonic antigen from the cholangiocarcinoma cell line, SLU-132, a mechanism that induces a decrease in the growth of this cholangiocarcinoma cell line in the nude mouse (45). Manipulation of endogenous cholecystokinin levels may be an important approach for the autocrine regulation of the growth of human cholangiocarcinoma (27).

Neuroendocrine Endocrine Regulation of Cholangiocarcinoma Growth

Cholangiocarcinoma displays characteristic features of neuroendocrine tumors such as the expression of chromagranin A and neuron-specific enolase as seen in Fig. 1 (2). The neurotransmitter dopamine is overproduced and secreted at increasingly high levels in cholangiocarcinoma (16). This study demonstrated that the enzymes that regulate dopamine synthesis are dysregulated in cholangiocarcinoma compared with normal cholangiocytes, causing cancerous cells to produce increased levels of dopamine (16). The study has also shown that blockade of dopamine production and secretion induces a decrease in cholangiocarcinoma growth in vitro and in vivo (16). A similar study has shown that the rate-limiting enzyme responsible for serotonin metabolism, tryptophan hydroxylase I, is upregulated and the enzyme responsible for degradation is markedly suppressed (2). Blocking tryptophan hydroxylase I proved to decrease tumor cell growth in models of cell culture and xenograft tumors (2).

The H3 histamine receptor agonist, RAMH, had been shown to inhibit cholangiocarcinoma growth (34). Treatment with RAMH induced a decrease in all cholangiocarcinoma cell lines but had no effect in normal cholangiocytes (34). RAMH inhibition of cholangiocarcinoma growth was associated with an increase in intracellular IP3 levels and protein kinase C-α translocation and a decrease in the expression of VEGF-A and VEGF-C and its receptors, VEGFR-2 and VEGFR-3 (34). Representative immunohistochemistry images of this expression are found in Fig. 2.

Other Neurotransmitters

Besides the above-mentioned neuroendocrine factors, there is increasing evidence that cholangiocarcinoma growth can be regulated by a number of other neuroregulatory molecules such as the inhibitory neurotransmitter, GABA (30), various endocannabinoids (23, 24, 32), and opioids (57).

GABA. Administration of GABA to cholangiocarcinoma cells and in tumors decreased proliferation and migration in a
dose-dependent manner (30). This effect was associated with increased intracellular IP3 and cAMP levels and subsequent activation of PKA and dephosphorylation of ERK1/2 (30).

**Endocannabinoids.** We have shown the differential effects of anandamide (AEA) and 2-arachidonyl glycerol (2-AG) on cholangiocarcinoma growth in vitro (24). The growth promoting effects of 2-AG was found to be via a cannabinoid receptor-independent mechanism involving the disruption of lipid raft structures in the cell membrane (24). Conversely, the antiproliferative actions of AEA were via a mechanism involving the stabilization of lipid rafts in the plasma membrane and the recruitment of death receptor complexes (24). Furthermore, we have shown that AEA suppresses tumor growth in vivo using a xenograft model of cholangiocarcinoma (23) and that there was a concomitant activation of the noncanonical Wnt pathway via upregulation of Wnt 5a (23). More recently, we have demonstrated that AEA suppresses tumor growth in vitro using a xenograft model of cholangiocarcinoma (23) and that there was a concomitant activation of the noncanonical Wnt pathway via upregulation of Wnt 5a (23). More recently, we have demonstrated that the antiproliferative actions of AEA are also associated with an increase in Notch 1 expression and activation, whereas the growth-promoting effects of 2-AG can be associated with an increase in Notch 2 expression and activation (32). The cross talk between the Wnt and Notch signaling pathways after AEA treatment and the reliance of these pathways on lipid raft structures are yet to be determined.

**Other Regulatory Peptides**

**Leptin.** Leptin is a hormone that is produced by the adipose tissue to regulate caloric homeostasis, is increased in obese patients (95), and stimulates the growth of a number of tumors (35). It has been proposed that the increased production of such a hormone by adipose tissue could explain the well-known correlation between obesity and increased incidence of various types of tumors (35). Administration of leptin increased the proliferation and the metastatic potential of cholangiocarcinoma cells in vitro through signal transducers and activators of transcription 3-dependent activation of ERK 1/2 (28). Leptin increased the growth and migration and was antiapoptotic for cholangiocarcinoma cells (28). Moreover, the loss of leptin function reduced the development and the growth of cholangiocarcinoma in an experimental carcinogenesis model induced by thioacetamide administration to rats (28).

**Endothelins.** Endothelins (ET) are vasoactive peptides that exert their effects through either ET\textsubscript{A} or ET\textsubscript{B} receptors (5). These receptors are overexpressed in cholangiocarcinoma cells (56), suggesting that the endogenous opioid system is somehow dysregulated during cholangiocyte-to-cholangiocarcinoma transformation. Associated with these growth promoting effects of μOR activation was an increase in ERK1/2, PI3-kinase and Ca\textsuperscript{2+}-dependent CamKII\textsubscript{α} pathways (57).
Administration of ET-1 to cholangiocarcinoma cells inhibited proliferation both in vitro and in vivo.

Microenvironment

Neoplastic epithelial cells coexist with a biologically complex stroma composed of various types of stromal cells as well as extracellular matrix, both of which create the complexity of the tumor microenvironment (73). Mouse models of tumorigenesis have revealed that stromal cells, in particular inflammatory cells, vascular cells, and fibroblasts, actively support tumor growth (11, 17, 70, 92). The role of the microenvironment in the regulation of cholangiocarcinoma growth, metastasis, and progression is largely unknown; however, what is known is summarized below.

Stromal fibroblasts. Under normal physiological conditions, fibroblasts have a low proliferative index and only secrete factors needed to maintain normal tissue homeostasis (6, 94). Indeed, normal fibroblasts provide biochemical cues that constrain epithelial tumor cells within their basement membrane (6, 94). In contrast, when homeostasis is disrupted during tissue injury, stromal cells rapidly and reversibly alter their phenotype and proliferation rate (94). However, during tumorigenesis, the fibroblastic wound healing machinery lacks the regulatory mechanisms to revert to normal homeostasis (94). The inability to downregulate the wound healing response affects stromal dynamics. Tumor-dependent changes in signaling and plasticity of the stroma trigger a continuum of alterations yielding a “primed” stroma that can support and incite tumor initiation or progression (94). Fibroblast activation protein is expressed by tumor-associated fibroblasts and fibroblasts involved in wound healing but not in normal fibroblasts (78). In cholangiocarcinoma, stromal fibroblasts have been shown to play an important role in migration and invasion of tumor cells (67, 68). Invasion of cholangiocarcinoma cells has been shown to increase after incubation with the supernatant from fibroblast cultures (67). The secreted factor thought to be

![Fig. 2. H3 histamine receptor agonist RAMH (10 mg/kg body wt) administration to mice bearing xenografted cholangiocarcinoma tumors decreased the expression of VEGF-A, VEGF-C, VEGF receptor (VEGFR)-2, and VEGFR-3 compared with vehicle-treated (0.9% NaCl) mice. Original magnification x20. A, C, E, G: vehicle. B, D, F, H: RAMH. Reproduced from Francis et al. (34) with permission from American Association of Cancer Research.](http://ajpgi.physiology.org/)
involved in this process is stromal-derived factor-1 (68), which is released from stromal fibroblasts and stimulates invasion and migration of the cholangiocarcinoma cells via interaction with the chemokine receptor CXCR4 (68). A schematic diagram of this can be found in Fig. 3.

**Angiogenesis**

The physiological process of the formation of new blood vessels from preexisting blood vessels is termed angiogenesis. A recent immunohistochemical analysis of microvessel density and lymphatic microvessel density revealed that intrahepatic cholangiocarcinoma tumors showed an induction of tumor-associated angiogenesis and lymphangiogenesis (89). Furthermore, tumors with increased microvessel density and lymphatic microvessel density were correlated with a higher recurrence rate, lower 5-yr survival rates, and increased nodal spread influencing patient survival (89). In addition, VEGF-A has been shown to play a role in the neovascularization of extrahepatic cholangiocarcinoma (62). The factors that drive angiogenesis including VEGF have also been shown to have distinct effects on cholangiocarcinoma growth in an autocrine manner (36, 37, 62, 86, 101, 105) as described above.

**Novel Treatment Regimes**

As mentioned previously, cholangiocarcinoma is relatively resistant to most currently approved chemotherapeutic agents, and as such these treatment options, although perhaps extending the prognosis for a few months, are largely ineffectual in curing this disease. Recently a number of experimental treatment options have come to the forefront. The most promising of these appears to be a multiple kinase inhibitor, sorafenib (98). This has been approved for use as a chemotherapeutic agent for renal cancer (76). Recently, sorafenib was shown to display significant tumor suppression in a rodent model of cholangiocarcinoma (12) and is currently undergoing phase II trials (9).

Another strategy to improve the treatment options of cholangiocarcinoma is to increase the sensitivity of cholangiocarcinoma to common chemotherapeutic agents. A large body of research has focused on plant-derived polyphenols, such as resveratrol (13, 65, 80), caffeic acid (71), tannic acid (66), and green tea polyphenols (53), as therapeutic and chemopreventive agents. Research indicates that these polyphenols may have antioxidant characteristics with potential health benefits including reducing the risk of cancer (83). Caffeic acid, a polyphenol extracted from the propolis of honeybee hives, exerts antiproliferative effects on cholangiocarcinoma (71) and other tumor types (22, 99) in vitro and in vivo by inhibiting the nuclear factor-κB pathway (71). Furthermore, high concentrations of resveratrol have been shown to be antiproliferative in a cholangiocarcinoma cell line (80) as well as in other tumor types (10, 21, 87) through a number of different mechanisms including increased cyclooxygenase 2 expression (87), facilitation of death receptor complex formation (20, 21), or cell cycle arrest (10).

Although it is interesting that these food-derived polyphenols exert antiproliferative effects on tumor growth in their own right, often the concentrations required are prohibitive for these compounds to be considered as viable treatment options. A more rational approach is to study the efficacy of these compounds as adjunct therapies to existing chemotherapeutic strategies. For example, tannic acid and the green tea polyphenol epigallocatechin-gallate have been shown to sensitize cholangiocarcinoma to chemotherapy-induced apoptosis in vitro (53, 66). Furthermore, the green tea polyphenol administered together with gemcitabine not only slowed cholangiocarcinoma tumor progression in a xenograft mouse model, it also decreased tumor volume (53). We have recently shown that resveratrol has a similar sensitizing effect on cholangiocarcinoma to the chemotherapeutic agents gemcitabine, 5-fluorouracil, and mitomycin C, potentially due to the suppression of the xenobiotic metabolizing enzyme cytochrome P-450 1b1 (33).

Another treatment option currently being explored involves the use of a neoadjuvant therapy regime followed by liver transplant (77). Historically, the surgical treatment of cholangiocarcinoma arising in patients with primary sclerosing cholangitis produced disappointing outcomes with no significant improvement of survival over those transplanted patients with primary sclerosing cholangitis alone (1, 39, 61, 79). Recently, the use of various neoadjuvant therapies prior to transplantation has given promising results (77). The established protocol initially involved rigorous patient selection criteria, excluding such parameters as the presence of a mass below the level of the cystic duct and evidence for intrahepatic or extrahepatic metastasis (77). Patients who had transperitoneal biopsy or violation of the bile duct during a prior attempt at surgical resection were also disqualified because peritoneal seeding has been encountered (77). Once selected, the patients commenced the neoadjuvant therapy regime of 4,000–4,500 cGy, administered by external beam radiation, followed by transcatheter radiation (2,000–3,000 cGy) with iridium-192 wires (77). In parallel, 5-fluorouracil was infused during the radiation treatment and followed by oral capecitabine until the day of transplantation (77). Prior to transplantation, the patients underwent a staging abdominal explorative operation to further screen for intrahepatic metastases, or lymph nodes and peritoneal metastases, and if no indication of metastases was present, transplantation was then performed. The results of this treatment regimen have been encouraging, with the 1-, 3-, and 5-yr survival rate after transplantation being higher than that after resection alone (77).

**Conclusion and Future Perspectives**

From the work described above it is obvious that there are large gaps in our knowledge concerning both the etiology and
progression of cholangiocarcinoma. Taken together with the increasing incidence of cholangiocarcinoma worldwide, the lack of effective treatment options is concerning. Increased efforts are needed to design multifaceted approaches to target key features of this complicated and resistant tumor. In addition, research into the mechanism of chemotherapeutic resistance and methods to decrease this resistance in these tumors may prove valuable in designing adjunct therapies.

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Because of editorial constraints, citations are rather illustrative than comprehensive, with a strong emphasis on review articles. We apologize to all investigators whose groundbreaking work could not be cited.

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References


43. Hidalgo M, Amador ML, Jimeno A, Mezzadra H, Patel P, Chan A, Johnson FE. Effect of cholecystokinin on human cholangiocarcinoma growth by 10.220.33.6 on June 22, 2017 http://ajpgi.physiology.org/ Downloaded from...


