

Cholangiocarcinoma, gone without the Wnt?

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Abstract

Cholangiocarcinoma (CCA) is a relatively rare malignancy of the intra- or extra-hepatic bile ducts that is classified according to its anatomical localization as intrahepatic, perihilar or distal. Overall, CCA has a dismal prognosis due to typical presentation at an advanced irresectable stage, lack of effective non-surgical treatments, and a high rate of disease recurrence. CCA frequently arises on a background of chronic liver inflammation and cholestasis. Chronic inflammation is accompanied by enhanced cell turnover with generation of additional inflammatory stimuli, and a microenvironment rich in pro-inflammatory mediators and proliferative factors that enable accumulation of mutations, transformation and expansion of mutated cells. A recent study by Boulter *et al* implicates the Wnt signaling cascade in cholangiocarcinogenesis. Wnt ligands Wnt7B and Wnt10A were found to be highly overexpressed in human CCA tissue. Wnt7B protein was present throughout the tumor stroma, and often co-localized with a subset of CD68⁺ macrophages. To address in a direct manner whether Wnt signaling is engaged in development of CCA, Boulter *et al* explored the Wnt signaling pathway in an experimental model that recapitulates the multi-stage progression of human CCA. Wnt ligands found to be elevated in human CCA were also upregulated during the course of CCA development following thioacetamide treatment. Wnt10a increased during the (pre-cancerous) regenerative phase, while Wnt7b induction paralleled tumor growth. Along with upregulation of target genes, the findings demonstrate that the canonical Wnt pathway is progressively activated during cholangio-carcinogenesis. Macrophage depletion, eliminating a major source of Wnt7b, prevented activation of the canonical Wnt cascade, and resulted in reduced number and volume of tumors in this model. Moreover, specific inhibitors of the canonical Wnt pathway (ICG-001 and C-59) caused reduction of tumor area and number, in xenograft and thioacetamide models of CCA. The aggregated findings show that experimental, and presumably human CCA, is a Wnt-driven tumor. Modulation of Wnt signaling, alone or in combination with surgical

or chemotherapy approaches, holds promise in the management of this fatal malignancy.

Key words: Intrahepatic cholangiocarcinoma; Liver neoplasms; Carcinogenesis; Wnt signaling pathway; Wnt7B protein; Wnt proteins

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Core tip: Cholangiocarcinoma (CCA) is a relatively rare malignancy of the intra- or extra-hepatic bile ducts with dismal prognosis. CCA frequently arises on a background of chronic liver inflammation and cholestasis, which creates a microenvironment rich in pro-inflammatory mediators and proliferative factors that enable accumulation of mutations, transformation and expansion of mutated cells. A recent elaborate study by Boulter *et al* (*J Clin Invest* 125:1269) has provided novel insights into the molecular pathogenesis of CCA. Involvement of the Wnt signaling pathway in cholangiocarcinogenesis, and effect of Wnt inhibitors on CCA development *in vivo* are discussed in this Editorial.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a relatively rare malignancy of the intra- or extrahepatic bile ducts that is classified according to its anatomical localization as intrahepatic, perihilar or distal^[1,2]. CCA accounts for 10%-20% of the primary liver malignancies, with perihilar (50%-67%) and distal extrahepatic tumors (27%-42%) comprising the majority of CCA cases^[1]. Tumor biology (*e.g.*, growth pattern, mutation spectrum) and clinical presentation, management and outcomes are different for the three CCA types. Overall, CCA has a dismal prognosis due to typical presentation at an advanced irresectable stage, lack of non-surgical potentially curative treatments, and a high rate of disease recurrence. The five-year survival rate is 5%-10%.

Our understanding of the molecular pathogenesis of CCA is limited. CCA frequently arises on a background of chronic liver inflammation and cholestasis, as reflected by risk factors of cholangiocarcinogenesis (*e.g.*, liver cirrhosis, viral hepatitis, hepatolithiasis, liver fluke infestation, primary sclerosing cholangitis). Chronic inflammation is accompanied by enhanced cell turnover with generation of additional inflammatory stimuli, and a microenvironment rich in pro-inflammatory mediators and proliferative factors that enable accumulation of mutations, transformation and expansion of mutated cells^[3,4]. Cholestasis may contribute to cholangiocarcinogenesis through effects of (conjugated)

bile salts on proliferation and invasion of cholangiocytes^[5,6].

The overall incidence of CCA has increased over the past decades and this is attributed to a global rise in the incidence of intrahepatic CCA. Liver transplantation is generally not considered for treatment of CCA due to frequent tumor recurrence and poor five-year survival rates after liver transplantation for intrahepatic CCA. Hence, resection is the only potentially curative treatment of CCA. The majority of patients with CCA, however, do not qualify for surgery and have to resort to palliative therapies. Molecular-targeted therapies hold potential for personalized treatment of malignancies including CCA^[7]. A recent study by Boulter *et al*^[8] implicates the Wnt signaling cascade in cholangiocarcinogenesis. Importantly, specific inhibitors of this pathway prevented tumor development in animal models of CCA.

THE WNT SIGNALING CASCADE

Wnt signaling is initiated by binding of membrane-bound Wnt ligand to a transmembrane receptor of the Frizzled family, and can operate in autocrine and paracrine modes^[9-11]. Wnt ligands are a family of secreted glycoproteins that have undergone a lipid modification (Cys-palmitoylation) that is essential for biological activity. The Frizzled family are G-protein coupled receptors that, alone or in conjunction with co-receptors (*e.g.*, Lrp5/6), serve as binding sites for Wnt ligands. Canonical Wnt signaling results in a transcriptional response in which the transcription factor β -catenin plays a central role, whereas non-canonical Wnt signaling cascades control the cytoskeletal structure or intracellular Ca^{2+} content through β -catenin-independent non-genomic actions. The canonical Wnt signaling pathway is the focus of the studies of Boulter *et al*^[8]. In the absence of Wnt signaling, β -catenin is targeted for proteasomal degradation by a multi-protein complex. Formation of this degradation complex is abrogated by activation of Wnt signaling, resulting in cytoplasmic accumulation and subsequent nuclear translocation of β -catenin. There, β -catenin acts in concert with other transcription factors (*e.g.*, TCF/LEF family members) to activate expression of target genes including cell cycle-related genes (*e.g.*, *CCND2*, *CDKN2A*).

Wnt signaling was identified through its role in carcinogenesis^[10], but not surprisingly found to participate in normal development and adult tissue homeostasis as well. Mutations in downstream components of the Wnt signaling pathway have been identified in various types of human cancers^[11,12]. For example, adenomatous polyposis coli (APC), a tumor suppressor that is part of the β -catenin degradation complex, is frequently mutated in colorectal and gastric cancers. Mutations in β -catenin that enhance protein stability, exemplifying a gain-of-function mutation, have been found in hepatocellular carcinoma^[11]. Hepatic adenomas that are positive for β -catenin have a high risk for malignant conversion and are typically resected, whereas other adenoma types are generally left untreated^[13]. Targeting of the Wnt

pathway is being explored as treatment of Wnt-driven malignancies^[14].

WNT SIGNALING IN CCA

By analyzing tumoral and matched unaffected liver tissue of patients with intrahepatic or perihilar CCA, Boulter *et al.*^[8] demonstrate that Wnt ligands Wnt7B and Wnt10A are highly overexpressed in tumor tissue. Wnt7B protein was present throughout the tumor stroma, often co-localizing with a subset of CD68⁺ macrophages. Moreover, tumors displayed elevated levels of transcripts of known β -catenin targets (*e.g.*, *CCND2*, *CDKN2A*, *BIRC5*), and cancerous biliary epithelium showed increased immunohistochemical positivity for a number of β -catenin targets. These findings suggest that canonical Wnt signaling is activated in human CCA.

To address in a direct manner whether Wnt signaling is engaged in development of CCA, this pathway was further explored in an experimental model that recapitulates the multi-stage progression (*i.e.*, chronic cholangiocyte damage, inflammation, biliary repair/regeneration, tumorigenesis) of human CCA. For this purpose, rats were treated with thioacetamide (TAA) and sacrificed at pre-cancerous (0-16 wk of treatment) and cancerous (20-26 wk of treatment) stages. Mirroring end-stage CCA in humans, strong nuclear β -catenin staining was observed in cancerous epithelium. In the pre-cancerous stage, regenerating ductules showed a membranous staining pattern. Wnt ligands found to be elevated in human CCA were also upregulated during the course of CCA development following TAA treatment. Wnt10a increased during the (pre-cancerous) regenerative phase, while Wnt7b induction paralleled tumor growth. Along with upregulation of target genes, above findings demonstrate that the canonical Wnt pathway is progressively activated during cholangiocarcinogenesis.

Through an elegant set of experiments Boulter *et al.*^[8] demonstrate that Wnt7B in tumor stroma is largely derived from recruited bone marrow-derived macrophages rather than from resident Kupffer cells, and that these cells play a key role in CCA progression. The role of macrophages in CCA growth was initially assessed in mice xenografted with three different human CCA cell lines. Groups of mice with established palpable subcutaneous tumors received vehicle or treatments to deplete phagocytic macrophages or prevent differentiation of monocytes into macrophages. Xenograft characteristics were determined after a further growth period of 3 wk. Loss of macrophages by either of the two strategies, resulted in reduced number of CD68⁺ macrophages and decreased Wnt7b expression in all xenografts. In two out of three xenografted CCA cell lines (*i.e.*, CC-LP-1 and SNU-1079, derived from intrahepatic CCA) this was accompanied by decreased expression of (human) proliferative genes, increased apoptosis and lowered tumor burden. The lack of functional consequences, despite loss

of Wnt signal, in xenografts derived from the third cell line (*i.e.*, WITT-1) may relate to its different origin (distal extrahepatic CCA) and/or distinct growth requirements. As a model more representative in terms of tumor micro-environment (stroma) and disease progression, Boulter *et al.*^[8] then studied the consequences of macrophage depletion (liposomal clodronate) in TAA-induced CCA. Strikingly, macrophage ablation prevented activation of the canonical Wnt cascade (loss of tumoral Wnt7B signal) and resulted in reduced number and volume of tumors.

The aggregated findings show that experimental, and presumably human CCA, is a Wnt-driven tumor. Since general macrophage depletion is not feasible in clinical practice, Boulter *et al.*^[8] explored the impact of specific inhibitors of the canonical Wnt pathway in the xenograft- and TAA-model of CCA. For this, they chose two targets that were elevated in human CCA, namely *CTBP1* and *PORCN*. *CTBP1* interacts with β -catenin to drive expression of growth-stimulating Wnt target genes, and their interaction can be targeted by ICG-001^[15]. As mentioned above, Wnt ligands require palmitoylation for biological activity and this lipid modification can be prevented by the *PORCN* inhibitor C-59^[16,17]. Both ICG-001 and C-59 were effective in reducing *in vitro* growth of five human CCA cell lines with presumed autocrine activation of canonical Wnt signaling (constitutive Wnt7B and β -catenin expression). Similar to macrophage ablation, ICG-001 and C-59 reduced tumor volume and mass in two CCA cell lines of intrahepatic origin when xenografted in mice, but did not affect WITT-1 xenograft growth. The reliance on Wnt signaling for proliferation and survival of CCA cells was confirmed in TAA-induced CCA, with ICG-001 and C-59 causing reduction of tumor area and number. Importantly, neither treatment affected body weight or caused liver test abnormalities, side effects observed with use of earlier generation Wnt inhibitors^[14].

PERSPECTIVE

The work of Boulter *et al.*^[8] demonstrates that the canonical Wnt pathway is activated in intrahepatic and perihilar CCA. Inhibition of canonical Wnt signaling, either by depleting the macrophage source of Wnt ligand or *via* pharmacological blockage, reduces CCA formation in a rat model that closely resembles human CCA. This is achieved through stimulation of apoptosis and reduced cell cycle entry. Thus, Wnt signaling is important for proliferation and survival of CCA cells in the TAA-model. It remains to be determined whether human CCA growth/progression is Wnt-dependent, and hence amenable to targeting by Wnt pathway inhibitors. More detailed insight into the interaction of Wnt signaling with the complex cellular surrounding, and its integration with other cellular signaling cascades, is warranted. Time will tell if systemic or CCA-directed Wnt inhibition, alone or in combination with surgical or chemotherapy approaches, will improve clinical outcomes of this fatal malignancy.

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