# Interleukin-6 Contributes to Mcl-1 Up-regulation and TRAIL Resistance via an Akt-Signaling Pathway in Cholangiocarcinoma Cells

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***Background & Aims:*** **Cholangiocarcinomas often arise within a background of chronic inflammation suggesting that inflammation imparts survival signals to this cancer. Previous studies have also shown that the inflammatory cytokine interleukin (interleukin [IL]-6) contributes to survival signals in an autocrine fashion and that myeloid cell leukemia-1 (Mcl-1), an antiapoptotic member of the B-cell leukemia-2 family, is an important participant in tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) resistance in this neoplasm. The present study evaluated the possibility that IL-6 signaling contributes to Mcl-1 up-regulation in cholangiocarcinoma.** ***Methods:*** **Protein kinase B (Akt) and Mcl-1 expression in human tissue was assessed by immunohistochemistry. The relationship between IL-6 signaling, Akt activity, and Mcl-1 expression was examined in cell lines.** ***Results:*** **Immunohistochemistry showed that the serine/threonine kinase Akt and Mcl-1 are strongly expressed in the preneoplastic bile duct inflammatory disease primary sclerosing cholangitis and in human cholangiocarcinoma specimens. Immunoblotting showed that Akt is expressed and constitutively phosphorylated in 3 human cholangiocarcinoma lines. Further analysis showed that treatment with anti–IL-6–neutralizing antiserum led to reduced Akt phosphorylation, diminished Mcl-1 expression, and enhanced TRAIL sensitivity. Likewise, the Akt inhibitor A443654.3 led to diminished signaling through the Akt pathway, decreased Mcl-1 expression, and enhanced TRAIL-mediated apoptosis.** ***Conclusions:*** **These findings not only show that an autocrine IL-6/Akt signaling pathway enhances Mcl-1 expression in cholangiocarcinoma but also suggest a strategy for overcoming the resulting apoptosis resistance.**

Cholangiocarcinoma is a highly malignant cancer that originates from bile duct epithelial cells. About 4000–5000 cases of cholangiocarcinoma are diagnosed annually in the United States, and the incidence of this malignancy is increasing.[1–3] Although surgical resection and transplantation are potentially curative, more than two thirds of patients present with advanced disease that is not amenable to these therapies. Moreover, the majority of patients undergoing surgical resection develop recurrent disease.[4] Unfortunately, there is no proven medical therapy for this neoplasm. Future therapeutic strategies, whether used in an adjuvant or primary setting, will require an understanding of the specific molecular pathways responsible for this cancer's growth, survival, and dissemination.

Cholangiocarinoma frequently arises in the background of chronic inflammatory diseases of the biliary tree.[3,5] For example, the risk of cholangiocarcinoma in patients with primary sclerosing cholangitis, a chronic inflammatory disease of the extra- and intrahepatic ducts, is approximately 8%–12%.[6–8] These data raise the possibility that inflammatory mediators might participate in the genesis and progression of cholangiocarcinoma, as has been suggested for several other malignancies.[9]

A candidate inflammatory mediator implicated in the biology of cholangiocarcinoma is interleukin (IL)-6. This cytokine is not only generated by inflammatory cells but also produced directly by cholangiocytes in response to inflammatory stimuli.[10] Moreover, cholangiocarcinoma cells autonomously secrete IL-6, which can then signal in an autocrine or paracrine manner.[11,12] Further studies have shown that IL-6 generates survival and mitogenic signals in cholangiocarcinoma cells.[11,12] Because these signals can circumvent anticancer therapies designed to selectively trigger tumor cell apoptosis, information re-

*Abbreviations used in this paper:* Akt, protein kinase B; Bcl-2, B-cell leukemia-2; CCA, cholangiocarcinoma; gp80, glycoprotein 80; gp130, glycoprotein 130; GSK-3, glycogen synthase kinase-3; IL, interleukin; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia-1; NF-κB, nuclear factor kappa B; PBS-T, phosphate-buffered saline 0.05%Tween; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PSC, primary sclerosing cholangitis; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.



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garding the specific antiapoptotic pathways activated by IL-6 in cholangiocarcinoma cells may help guide future therapies.

The phosphatidylinositol 3-kinase (PI3K)/Akt cell–signaling pathway is responsible for potent survival signals in many cell types.[13] This pathway is activated by a diverse group of plasma membrane receptors after binding of their cognate ligands.[13] Because of its ability to suppress proapoptotic stimuli, this pathway has been strongly implicated in carcinogenesis.[14–16] Akt, an effector kinase in this pathway, is itself activated by PDK1-mediated phosphorylation. After activation, Akt inhibits apoptosis[17] by not only inactivating proapoptotic molecules such as B-cell leukemia-2 (Bcl-2)–associated x protein,[18] Bcl-XL/Bcl-2–associated death promoter,[19,20] glycogen synthase kinase-3 (GSK-3),[21] and the transcription factor forkhead in rhabdomyosarcoma[22] but also activating downstream antiapoptotic modulators such as nuclear factor kappa B (NF-κB),[23] X-linked inhibitor of apoptosis,[24] mammalian target of rapamycin,[25,26] and the p53 E3 ubiquitin ligase MDM2.[27] Although enhanced Akt expression has been reported in cholangiocarcinoma,[28,29] it is unclear whether Akt participates in IL-6–induced survival signals in this cancer. Furthermore, because several Akt inhibitors are being developed for anticancer therapy, preclinical information is necessary regarding the potential effects of Akt inhibition in cholangiocarcinoma cells.

Survival pathways ultimately impact on the core apoptotic machinery to block cell death. Bcl-2 family members play a critical role in modulating and regulating apoptosis at the level of the mitochondria.[30,31] Although a large number of antiapoptotic Bcl-2 proteins have been identified,[32]myeloid cell leukemia-1 (Mcl-1) appears to be a particularly important antiapoptotic protein expressed in cholangiocytes and cholangiocarcinoma.[33] Mcl-1 is unique among Bcl-2 family members in having a proline, glutamate, serine, threonine rich (PEST) sequence that targets it for rapid turnover by the proteosome.[34] As a consequence, Mcl-1 has a very short half-life (<4 hours)[35] and is highly regulated at the levels of transcription, translation, and posttranslational modification.[36,37] Although Akt has been implicated in the regulation of Mcl-1,[38] the cellular mechanisms responsible for this regulation remain obscure.

The overall objective of this study was to examine the relationship between IL-6 secretion, Akt activation, and Mcl-1 expression in human cholangiocarcinoma. To address this objective, the following questions were formulated: (1) Are Akt and Mcl-1 up-regulated in the preneoplastic biliary disease PSC and human cholangiocarcinoma? If so, is the up-regulation IL-6 dependent? (2) Does IL-6 enhance Mcl-1 expression via an Akt-dependent or -independent mechanism? (3) How does Akt regulate Mcl-1? and (4) Does Akt inhibition sensitize cholangiocarcinoma cells to apoptosis? Results of the present study not only show that signaling through an IL-6/Akt pathway up-regulates Mcl-1 in cholangiocarcinoma cells through transcriptional and posttranslational mechanisms but also show the interruption of IL-6/Akt signaling using 2 different strategies results in enhanced sensitivity of cholangiocarcinoma cells to the cytotoxic cytokine tumor necrosis factor–related apoptosis-inducing ligand (TRAIL).

## Experimental Procedures

### Human Liver Tissue

After approval by the Mayo Clinic Institutional Review Board, immunohistochemical analysis was performed on 77 archived surgically resected liver specimens. The liver specimens were obtained from explanted livers of liver transplant recipients undergoing liver transplantation for end-stage hepatitis C (n = 7) and primary sclerosing cholangitis (PSC, n = 29). Cholangiocarcinoma specimens (CCA, n = 33) were obtained from 8 liver transplant recipients and 25 surgical resections. Normal liver specimens were obtained from 25 liver biopsy samples of 17 living and 8 cadaveric liver transplant donors. Patients with other potential etiologies for their liver disease were excluded from the study by examination of the medical record and re-review of the pathologic specimens.

### Immunohistochemistry

For placement on glass slides, paraffin-embedded liver tissue was cut (5 μm thick), deparaffinized, and hydrated. Antigen retrieval was performed by boiling the specimen in 1 mmol/L EDTA (pH 8.0) for 40 minutes in a commercial vegetable steamer. After cooling to room temperature for 20 minutes, slides were washed in running distilled water for 5 minutes, soaked in 3% hydrogen peroxide for 10 minutes and incubated in blocking buffer (5% normal goat serum in phosphate-buffered saline 0.05%Tween [PBS-T]) for 30 minutes at room temperature. Immunohistochemistry was performed using a polyclonal rabbit anti-Akt antibody (Upstate, Lake Placid, NY) and a polyclonal rabbit anti-Mcl-1 antibody (S-19 Ab, Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The samples were incubated with primary antibody dilutions of 1:250 (Akt) and 1:1000 (Mcl-1) in blocking buffer overnight at 4°C. As negative controls, the primary antibody was replaced with normal goat serum. After extensive washing with PBS-T, the sections were