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TOPIC HIGHLIGHT

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JAK-STAT pathway in carcinogenesis: Is it relevant to cholangiocarcinoma progression?

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Abstract

The features of JAK-STAT signaling in liver cells are discussed in the current review. The role of this signaling cascade in carcinogenesis is accentuated. The possible involvement of this pathway and alteration of its elements are compared for normal cholangiocytes, cholangiocarcinoma predisposition and development. Prolactin and interleukin-6 are described in detail as the best studied examples. In addition, the non-classical nuclear translocation of cytokine receptors is discussed in terms of its possible implication to cholangiocarcinoma development.

Key words: Cholangiocarcinoma; Janus tyrosine Kinases; Signal Transducers and Activators of Transcription; Prolactin; Interleukin-6; Cytokine receptors; Receptor tyrosine kinases

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INTRODUCTION

The signaling pathway of Janus tyrosine Kinases-Signal Transducers and Activators of Transcription (JAK-STAT) is activated by a variety of hormones (prolactin, growth hormone, leptin, erythropoietin), as well as cytokines, and growth factors *via* their receptors. JAK-STAT signaling participates in the regulation of cell proliferation, differentiation, survival, motility, and apoptosis in different organs including liver[1]. During the last decades, the data on cytokine/growth factor receptors expression and functions in different liver cell types (hepatocytes, cholangiocytes, Kupffer and stellate cells) have rapidly grown. It highlights the importance of JAK-STAT signaling in normal liver physiology and pathology. Moreover, deregulation of this pathway is closely associated with tumorigenesis.

Cholangiocarcinoma is a malignancy of the biliary epithelium with the worst prognosis among other liver tumors. It is relatively rare, but it frequently progressively increases. Molecular cholangiocarcinoma markers are mainly nonspecific and require further investigation. The main risk factors for cholangiocarcinoma development are hyperplasia of bile duct epithelia induced by bile duct obstruction, hepatolithiasis, inflammation, fibrosis, and some other factors[2-4]. Though the role of the JAK-STAT cascade is poorly deciphered both in normal cholangiocytes and in cholangiocarcinoma cells, a number of papers have been published recently where the role of prolactin (Prl), growth hormone (GH) and interleukin-6 (IL-6) and expression of corresponding receptors in cholangiocytes has been shown[5-7]. Expression of these receptors undergoes essential changes under hyperplasia of bile duct epithelia induced by bile duct obstruction, thus the role of the JAK-STAT pathway should attract further investigations in this area.

PRINCIPLES OF JAK-STAT SIGNALING

The main site for JAK-STAT signaling initiation is the cellular membrane; however, internalization pathways have been recently shown to be also important for the generation of comprehensive biological effect and sustaining signal transduction. Among other intracellular targets of receptor-JAK-STAT the cascade nucleus plays a key role[8-10]. Mitochondrial targeting of some cytokine receptors (GH receptor) has also been described[11].

The initial steps of signaling cascade activation by hormone (cytokine) are still intensively studied, but for many of them an interaction between signaling molecules and preformed receptor dimers leads to rotation of intercellular parts of receptor and subsequent activation of receptor-associated JAKs[12] followed by STAT docking on receptor and its phosphorylation, dimerization, and nuclear translocation (Figure 1).

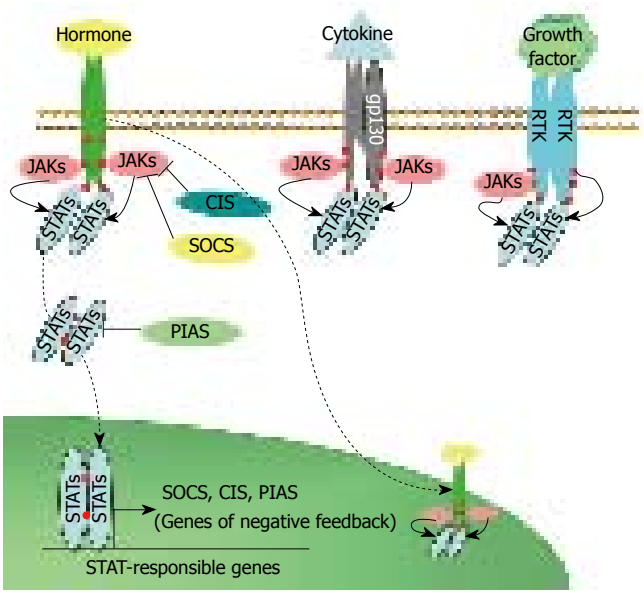

Figure 1 Schematic representation of JAK-STAT signaling induced by hormones, cytokines, and growth factors. RTK: Receptors tyrosine kinases; JAK: Jannus kinase; STA: Signal transducer and activator of transcription; SOCS: Suppressor of cytokine signaling; CIS: Cytokine induced suppressor; PIAS: Protein inhibitors of activated STATs. Solid arrows - phosphorylation; Dashed arrows - nuclear translocation; Blunt arrows - inhibition; Red circles - phosphorylated tyrosines.

Different JAKs (JAK1, JAK2, JAK3, and Tyk2) and STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) participate in JAK-STAT signal transduction. STAT3 and STAT5 are expressed in many cell types, activated by various hormones, cytokines and growth factors and play a role in different biological responses, whereas other STAT proteins (STAT1, 2, 4 and 6) are revealed mainly in specific cell types and participate predominantly in host defense mechanisms[13,14]. Phosphorylated STAT dimers can bind to STAT-responsive elements in the promoters of various genes, resulting in modulation of their transcription. STATs often act on gene transcription in cooperation or competition with NF-κB, AP-1, and a glucocorticoid receptor due to close co-localization of correspondent binding sites[15-17] accomplishing a crosstalk with other signaling cascades.

Suppressors of cytokine signaling (SOCS) proteins are able to inhibit JAK-STAT signaling. SOCS1 and SOCS3 are the most potent and broadly distributed suppressors with similar inhibition effects on gp130 related, Prl, and GH signaling. Promoter regions of SOCS1 and SOCS3 genes possess functional STAT binding elements and activation of JAK-STAT signaling induces rapid up-regulation of SOCS proteins by STAT-dependent pathways. In accordance with negative feedback mechanism JAK-STAT signaling is inhibited by SOCS proteins association with catalytic domains of JAKs as well as by SOCS binding to phosphorylated tyrosine residues of cytokine, GH, Prl, leptin, and erythropoietin (Epo) receptors which act as docking sites for downstream signaling[18-20]. STAT activation can also be inhibited by direct interaction with protein inhibitors of activated STAT proteins (PIAS)[21].

In some cases activation of STATs occurs independently of JAKs and is involved in receptor tyrosine kinase signaling. This activation is induced by direct interaction with receptor tyrosine kinases or by subsequent downstream signaling with implication of other kinases such as Src. For example, STAT3 is activated in response to stimulation by EGF, HGF, CSF-1, PDGF and other growth factors; and their corresponding receptor tyrosine kinases (ErbB1, ErbB-2, c-met, CSF-1, PDGF receptors, and others) possess a common STAT3 docking motif in their cytoplasmic domains. STAT5a phosphorylation is induced by EGF[13,22,23].

JAK-STAT SIGNALING AND CARCINOGENESIS

Altered activation of JAK-STAT signaling has been shown in multiple solid tumors and leukemia[24-26]. In different experimental models tumorigenesis is associated with increased expression and/or activity of JAK1, 2[27,28] or STAT3, 5[29]. A constitutively active mutant of STAT3 has been shown to transform rat fibroblasts[30], and the increased expression of wild type STAT5 in the lymphoid lineage induced T cell leukemia in mice[31]. A constitutively active mutant of JAK2 has been identified in patients with polycythaemia vera[32]. Moreover, increased levels of correspondent ligands can be associated with JAK-STAT induced carcinogenesis: in animal models of prostate and mammary gland hyperplasia with local overexpression of Prl[33,34]; in humans hyperprolactinemia is considered as a risk factor for breast and, probably, prostate cancer[35,36]; overexpression of GH in transgenic mice leads to increased frequency of mammary adenocarcinoma[37]. A particular and essential role for neoplasia progression of autocrine production of Prl has been revealed[34,38]. Only autocrine, but not exocrine GH, is suggested to promote neoplasia[39-41].

The role of the JAK-STAT cascade is not limited to induction of carcinogenesis: for example, the predominant role of TYK2 and STAT1 in many types of cancer is antitumorigenic *via* regulation of apoptosis[42]. Moreover, in some models JAK1 and 2 also play an antitumorigenic role[43]. Prl induced activation of JAK2 prevents breast cancer cells mesenchymal transition and acts as an invasion suppressor[44,45]. Reduced phosphorylation of STAT5 in breast cancer highlights patients with a worse prognosis of disease development[46].

CHOLANGIOCARCINOMA DEVELOPMENT AND JAK-STAT SIGNALING

A number of signaling molecules acting via the JAK-STAT pathway participate in regulation of bile duct cell functions. Among them, Prl and IL-6 (activation of complete JAK-STAT cascade), and EGF, HGF (activation of STAT *via* receptor tyrosine kinases) have their receptors in human and animal cholangiocarcinoma tissues and cell lines. Other JAK-STAT signaling molecules involved in the development of other cancer types have not been yet investigated.

Since the development of cholangiocarcinoma is closely related with obstructive, fibrotic, cirrhotic, and inflammation

changes of biliary epithelium leading to its hyperplasia or dysplasia, it is reasonable to compare the peculiarity of JAK-STAT signaling induced by different ligands in normal cholangiocytes, bile duct cells proliferating under conditions of different hepatic pathologies considered as a predisposition to cholangiocarcinoma development, and in cholangiocarcinoma cells.

HORMONAL INDUCTION OF JAK-STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

Prolactin signaling

Prl is a multifunctional pituitary hormone that also works as a locally produced cytokine. Prl regulates differentiation, proliferation, water-salt balance in different cell types including many ductal structures (such as ductal systems of mammary, lacrimal, submaxillary, prostate glands, pancreas, and kidney) and possesses high immunomodulatory activity[47,48]. Prl participates in the stimulation of rat bile duct cell proliferation and cholangiocyte regulation of bile water salt balance[49,50].

Prl receptor (PrlR) exists in several isoforms with a tissue specific ratio of expression. Long and short rat PrlR isoforms are the result of alternative splicing of cytoplasmic domain exons of a single gene. Several intermediate PrlR isoforms with varying cytoplasmic domains have been found in human normal and tumor tissues. The short PrlR isoform has been suggested to negatively regulate activity of the long isoform by heterodimerization. JAK-STAT pathway with predominant usage of JAK2 and STAT5 proteins is the main signaling pathway activated by Prl. STAT1 and STAT3 proteins may also be involved in Prl signaling with nuclear translocation of their homo- and heterodimers. SOCS1, 2, and 3 attenuate Prl signaling[47,48,51-53].

Prolactin JAK-STAT signaling and their alterations under conditions of cholangiocarcinoma predisposition and development

Systemic Prl levels are elevated under different liver pathologies associated with increased cholangiocarcinoma incidents[54-56], while local Prl production by the cholangiocarcinoma cell line (RS-1) is observed only in a few samples (Ostroukhova & Smirnova, unpublished observations).

Normal animal and human bile duct cells express PrlR messenger RNA and protein at a relatively low level. Contrary to hepatocytes where short PrlR isoform predominates, normal rat bile duct cells express a predominantly long receptor isoform that is similar for such Prl target tissues, such as the uterus, pituitary, and mammary gland[7,57].

Hyperplasia of bile duct epithelia in rats induced by bile duct obstruction or normally occurring in immature animals as a risk factor of cholangiocarcinoma development is characterized by sharp elevation of cholangiocyte PrlR expression in both males and females. Similarly, obstructive human liver diseases with jaundice development and

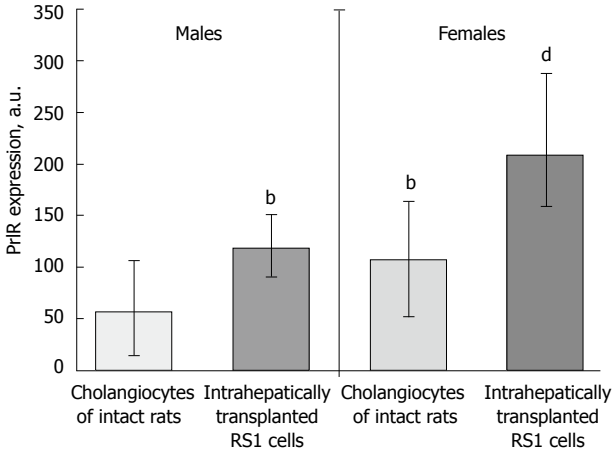

Figure 2 Increasing of prolactin receptor expression in rat liver transplanted RS-1 cholangiocarcinoma cell line. a.u, arbitrary units of intensity of PrlR expression. Medians, upper and lower quartiles. $^{b}P < 0.001$, *vs* intact males, $^{d}P < 0.001$, *vs* intact females.

enrichment of bile ducts are accompanied by a pronounced increase in cholangiocyte PrlR expression as compared with mild cholestasis without jaundice with limited affect on bile duct cell proliferating activity[57]. Obstructive conditions induce both dramatic elevation of the long receptor isoform and an increase in short isoform expression in bile duct cells. The level and isoform ratio of cholangiocyte PrlR in normal and obstructive conditions are only slightly dependent on gonadal hormones and independent of Prl[7,49,57].

Human cholangiocarcinoma samples are characterized by high PrlR expression significantly exceeding PrlR expression in cholangiocytes and hepatocytes under conditions of non-tumor liver diseases (Ostroukhova & Smirnova, unpublished observations). Intrahepatic transplantation of RS1 cholangiocarcinoma cells induces significant elevation of tumor cell PrlR expression (Figure 2) as compared both with subcutaneous tumor transplants and cholangiocytes of normal animals[58].

In cholangiocarcinoma cells the isoforms ratio is similar to the ratio observed in cholangiocytes proliferating under obstructive conditions. Surprisingly, relatively high levels of PrlR in transplantable cholangiocarcinoma have been revealed not only in female recipients, but also in male counterparts under conditions of relatively low levels of both Prl and estrogens[58]. Thus, it seems possible that the main regulators of PrlR expression in cholangiocarcinoma differ from sex hormones and may be connected with tumor derived autocrine/paracrine factors. Such unusual regulation may be an early event of cholangiocyte dysplasia since sharp elevation of PrlR expression in both males and females primarily occurs in bile duct cells proliferating in response to bile duct obstruction[7,49].

Prolactin - estrogen cross-talk and cholangiocarcinoma development

Prl as a female sex hormone acts in close connection with estrogens. In addition to systemic positive regulation of Prl production by estrogens and vise versa, a cross-talk between these hormones has been shown at the level of target tissues.

Prl stimulates both alpha and beta mRNA estrogen receptor expression in some tissues and putative STAT5α and STAT5β response elements have been identified in their promoters[59].

Estradiol *via* estrogen receptor (ER) alpha activates transcriptional expression of human PrlR through promoter Ⅲ that functions in different tissues including liver. Estrogens increase and tamoxifen reduces expression of Prl receptor mRNA in breast tumors and some other tissues[60-62]. Estrogens participate in regulation of downstream Prl signaling using different mechanisms. Estrogens can attenuate JAK-STAT signaling by Prl. Estrogens *via* ER α up-regulate SOCS2 expression thus diminishing JAK-STAT signaling. ER alpha activation could diminish PrlR signaling *via* direct interaction with STAT5α which leads to reduced STAT5α nuclear translocation. On the contrary, nongenomic action of estrogens that requires a ligand-binding domain of the membrane estrogen receptor alpha leads to rapid induction of STAT3 and STAT5 phosphorylation, their nuclear translocation, and transcriptional activity[61,63-66].

All these facts are very important taking into account that both PrlR and ERs are sharply increased in human cholangiocarcinoma samples and cell lines. While normal cholangiocytes express extremely low ERs, cholangiocarcinoma cells are characterized by intensive cytoplasmic and nuclear expression of ERα and ERβ as well as increased ERα/ERβ ratio that is typical for other cancer types. Besides, these estrogens potentiate and tamoxifen attenuates proliferation of human cholangiocarcinoma cell lines *in vitro*[4,6]. As in the case of PrlR, high level of ER expression in cholangiocarcinoma have been revealed not only in females, but also in males[67]. Based on both cytoplasmic and nuclear ER localization in cholangiocarcinoma cells it seems possible that estrogen regulation of Prl induced tumor JAK-STAT signaling is both nuclear and nongenomic and its bottom-line effects should be investigated carefully. It is interesting that in spite of high sensitivity of cholangiocarcinoma cells to both types of female sex hormones cholangiocarcinoma development is more frequent in men (1.5-1) than in women[68]. It may be due to attenuation of Prl tumor signaling by estrogens in the case of relatively high levels of both hormones in women. In any case combined therapy with tamoxifen and bromocriptine may be perspective clinical strategy for treatment of cholangiocarcinoma.

Cross-talk of Prl with EGF signaling leading to ErbB1 and ErbB2 phosphorylation has also been found, e.g. in breast cancer cells[69]. Since both Prl receptor and ERbB1 and ErbB2 are present in cholangiocarcinoma cells it may be relevant also to cholangiocarcinoma Prl signaling.

Growth hormone (GH) JAK-STAT signaling

As in the case of Prl, JAK-STAT signaling activated by GH mainly includes JAK2 activation and STAT5 phosphorylation, though STAT1 and 3 activation are sometimes also implicated. GH induces the expression of several negative regulators of GH signaling: SOCS1, 2, and 3, CIS[70,71].

GH receptor is present in normal rat cholangiocytes and isolated cholangiocytes respond to GH stimulation by IGF-1 secretion that in turn promotes cholangiocyt proliferation. GH receptor expression is increased in rat cholangiocytes after common bile duct ligation[72]. Contrary, in total liver of mice under the same conditions and humans with obstructive cholestasis and cirrhosis, changes of GH receptor expression and IGF-1 production are opposite[73]. GH signaling in cholangiocarcinoma cells has not been yet investigated.

Leptin JAK-STAT signaling

Leptin receptor is homologous to gp130 and can activate JAK2, mainly STAT3, but also STAT5, STAT6, STAT1 proteins[74-77]. SOCS3 is a negative regulator of leptin JAK-STAT signaling[18,78,79].

Autocrine/paracrine leptin production and leptin mRNA have been shown in different types of cancer cell lines *in vitro* and in some tumors *in vivo*. Serum leptin level also increases in patients with some cancer types[76,80]. Leptin is considered an agent responsible for overcoming the antiproliferative effect of antiestrogen therapy in different cancer types. Leptin receptors have been found in different cancer types and cell lines and leptin signaling is associated with suppression of tumor cell apoptosis[13,78].

Leptin expression has been detected in liver stellate cells and its local production is increased under obstructive cholestasis conditions. Leptin acts as a direct hepatic stellate cell survival agent[81-83]. Serum leptin is also elevated after common bile duct ligation and CCl_4 induced liver fibrosis that are both risk factors for cholangiocarcinoma development[84]. The leptin receptor has been found in bile duct cells of one type of mammalian species[85]. The role of leptin induction of JAK-STAT signaling needs to be investigated in cholangiocarcinoma development, and known STAT3 activation during cholangiocarcinoma progression (see below) may be partly due to leptin signaling.

Erythropoietin (Epo) JAK-STAT signaling

Epo is produced mainly by the fetal liver and the adult kidney. Epo interaction with Epo receptor activates JAK2 and STAT5[86,87]. Due to its erythropoietic functions, Epo has been widely used for prevention and treatment of cancer-associated anemia. However, Epo proangiogenic activity can compromise its beneficial effects in cancer patients.

Expression of Epo and Epo receptors has been found in a number of nonhematopoietic tissues as well as in different types of primary tumors and tumor cell lines suggesting the generation of autocrine-paracrine growth stimulatory Epo-Epo receptor loop in cancer cells. Experimental blockade of Epo signaling results in inhibition of different tumor growth and angiogenesis with concomitant elevation of apoptotic death of tumor and vascular endothelial cells[87-90].

Epo and/or Epo receptor expression have been found in liver stromal cells and some primary hepatic tumors, and tumor cell lines as well as in Kupffer cells during regeneration[87,89-93]. Still, we have not found any data on Epo expression and signaling in human cholangiocarcinoma

and its animal models. Based on the frequency of Epo and Epo receptor expression in different types of tumor cells, a key role of the fetal liver in Epo production, a role of hepatic progenitor cells, enriched in fetal liver, in liver cancer development as well as possibility of bone marrow derived liver stem cells to differentiate into biliary cells[90,94-97] it is easy to assume that the same situation occurs in cholangiocarcinoma development. Several facts support this assumption. In an earlier study the elevation of liver Epo production after bile duct ligation has been shown[98]. Data on the possibility of tissue specific estrogen modulation of local Epo expression together with elevation of estrogen receptor expression in human cholangiocarcinoma are also of great interest in this connection[6,86,99,100].

CYTOKINE INDUCTION OF JAK-STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

Interleukine-6 (IL-6) induced JAK-STAT signaling

IL-6 and other cytokines of this family have their own receptors, but need a common receptor subunit gp130 for signal transduction. Binding of IL-6 to its receptor leads to dimerization of gp130, resulting in activation of gp130-associated JAK kinases (JAK1, JAK2, and TYK2) and subsequent activation of STAT3 proteins forming homodimers or rarely heterodimers with STAT1. There is reciprocal negative regulation of JAK-STAT3 and MAPK signaling by IL-6[15,101,102]. SOCS3 activated by STAT3 suppresses IL-6 signaling[15,18-20].

Alterations of local and systemic IL-6 level under conditions of cholangiocarcinoma predisposition and development

Autocrine/paracrine production of IL-6 by normal liver bile duct cells as well as by cultured human intrahepatic biliary epithelial cells is very low. Bile duct obstruction triggers pronounced elevation of IL-6 mRNA and protein production by bile duct cells, which can be mediated by inflammatory conditions[103-105]. A pronounced autocrine/paracrine secretion of IL-6 by different human intrahepatic cholangiocarcinoma cell lines has been observed *in vitro*[106,107]. Obstructive changes as well as cholangiocarcinoma development are also associated with elevation of systemic plasma IL-6 level[108].

Alterations of IL-6 signaling under conditions of cholangiocarcinoma predisposition and development

In vitro normal bile duct cells express both IL-6 receptor and gp130 messenger RNA and protein, but their expression level is further increased in cholangiocarcinoma cells. Appearance of autocrine IL-6 production and high level of its receptors lead to autocrine/paracrine loop of IL-6 signaling during cholangiocarcinoma progression[5,109].

IL-6 target genes in cholangiocarcinoma progression

IL-6 enhances cell survival and proliferation of human malignant cholangiocytes by up-regulation of myeloid cell leukemia-1 (mcl-1) mRNA and protein expression which is a key member of the antiapoptotic Bcl-2 protein family[107,110]. Disruption of IL-6 signaling at different levels diminishes Mcl-1 promoter activity, its mRNA and protein level in human cholangiocarcinoma cell lines.

Sustained IL-6/JAK-STAT signaling and enhanced Mcl-1 expressions in cholangiocarcinoma are associated with at least partial epigenetic SOCS3 silencing. In human cholangiocarcinoma tissues and cell lines unusual inverse correlation exists between phosphorylated STAT-3 and SOCS3 protein expression. SOCS3 negative cholangiocarcinoma (as well as cholangiocarcinoma lines) samples are characterized by extensive methylation of SOCS3 promoter as compared with nontumor tissue. Treatment with demethylating agent restores IL-6 induction of SOCS3 leading to termination of phosphorylated STAT3 response, reduction of cholangiocarcinoma cells level of Mcl-1, and sensitization of cholangiocarcinoma cells to Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL)-mediated apoptosis[111,112].

GROWTH FACTOR INDUCTION OF STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

HGF induced STAT signaling

HGF is a growth factor acting *via* receptor tyrosine kinase c-Met and inducing matrix dissociation, motility of epithelial cells, and enhancing invasiveness of tumor cells. In the 5'-flanking region of human HGF gene cis-acting STAT element has been found. Interruption of STAT3 signaling by dominant-negative STAT3 reduces HGF promoter activity[113,114].

Interaction of HGF with its receptor c-Met leads to receptor dimerization and phosphorylation of intracellular receptor domain which forms the docking sites for interaction with multiple downstream signaling molecules including STAT3. STAT3 activation has been shown to be necessary for full biological responses of HGF and HGF induced proliferation[115-117]. In hepatocytes and some other cell types HGF activates the STAT3 signaling pathway, moreover, not only STAT3 but also STAT1α/STAT1β and STAT5 interact with c-Met[118,119]. STAT3 phosphorylation induced by HGF/c-Met signaling is limited by consequent positive HGF action on SOCS3 expression in some cell types. SOCS3 has been observed to act as a negative regulator of HGF-induced cell migration[120-122].

Alterations of local and systemic HGF level

In situ hybridization studies have demonstrated that there is a close paracrine ligand-receptor relationship between HGF expressed in mesenchymal cells and c-Met expressed in the adjacent epithelial cells. HGF production is stimulated by various cytokines including IL-6-type cytokines. Both HGF and c-met mRNAs and protein products are overexpressed in cholestatic animal liver and human hepatolithiasis specimens[123-125]. Serum HGF level is elevated in cholangiocarcinoma patients[126]. Cholangiocarcinoma cell lines do not secrete HGF, but

their co-cultivation with neutrophils induces a high level of HGF secretion by these mesenchymal cells and may enhance invasiveness of tumor cells[127].

Alterations of HGF signaling

c-Met is expressed in cultured mouse biliary epithelial cells and liver bile ducts[123,128]. c-Met activation and elevation of c-met mRNA and protein expression have been shown in mouse chemically induced cholangiocarcinoma, different cholangiocarcinoma cell lines, and human cholangiocarcinoma patients. c-Met overexpression is more prominent in induced cholangiocarcinoma in rats and human cholangiocarcinoma cell lines as compared to normal and hyperplastic intrahepatic biliary epithelium[3,5,126,129-131]. The presence of HGF-c-Met-STAT3 positive autocrine/paracrine loop in cholangiocarcinoma may confer increased survival, growth, and invasiveness during cholangiocarcinoma progression. The malignancy of cholangiocarcinoma cells containing activated STAT3 may be at least partly due to stimulation of HGF expression in adjacent mesenchymal cells and c-met overexpression in tumor cells.

ERbB2 induced JAK-STAT and STAT signaling

ErbB2 also known as HER-2 or HER-2/neu is a member of ErbB receptor tyrosine kinase family acting mainly as the most common partner for heteromerization for other members of this receptor family (ErbB1, ErbB3 and ERbB2) among which EGF is the main ligand for ErbB1 (EGFR). ErbB receptors may be constitutively associated with JAK kinases. Direct association between ErbB2 and other ErbB receptors with STAT3 has also been found. Src kinases are the other molecules for ErbB receptor mediated STAT signaling and potential upstream regulators of JAK kinases. Ligand induced receptor dimerization leads to recruitment of Src kinase that induces JAK and STAT3 phosphorylation, dimerization and nuclear translocation[132,133]. Neuregulin induced heterodimers of ErbB2 and ErbB4 may activate STAT5[23]. Expression of SOCS1, 3, 4, 5 is elevated by EGF treatment[134-136]. Tumor cell types that over express ErbB2 preferentially activate prosurvival STAT signaling. In different tumors an autocrine loop between cytokines and ErbB receptors induces constitutive STAT3 activation[133,137].

In human non-neoplastic hepatic tissue ErbB2 mRNA and protein products have been found only in large mature bile ducts or have not been revealed at all[138-140]. Human gallstone disease and common bile duct ligation in rats are accompanied by appearance or elevation of ErbB2 expression in cholangiocytes as compared with normal liver but its expression was lower than in cases of cholangiocarcinoma[138,141,142]. ErbB2 overexpression has been revealed in different human biliary tract cancer cell lines, xenobiotic induced rodent cholangiocarcinoma, as well as in human cholangiocarcinoma tissue samples. Overexpression of both ErbB1 (EGFR) and ErbB2 may serve as a base for enhancement of EGF signaling in cholangiocarcinoma. Altered ERbB2 expression occurs early in cholangiocarcinogenesis and may play an important role in its progression[129,130,139,143,144].

PERSPECTIVE THERAPEUTIC ADDRESSING OF THE JAK-STAT PATHWAY

As illustrated above, signaling of most hormones, cytokines and growth factors involved in cholangiocyte regulation seems to be enhanced under the progression of cholangiocarcinoma. It is associated, not only with increment of their systemic levels, but also (probably mainly) due to their local hepatic secretion and increment of the expression of their receptors. Taking together these observations we can suggest that local manipulation of JAK-STAT activation can be a very perspective therapeutic approach for managing of cholangiocarcinoma at different stages of its progression. Recent advances in development of hormonal and cytokine antagonists (recombinant proteins for Prl and GH and peptide for IL-6[145-147]) give us an efficient tool for this treatment. Since synthetic specific inhibitors of JAK and other tyrosine kinases are intensively developed, we could suggest their efficient application for managing of cholangiocarinoma in addition to antagonists.

For the moment, the most specific and efficient way to inhibit the components of the JAK-STAT pathway is local expression (tissue-specific) of dominant negative analogues of JAKs and STATs. Evidently, their application is now limited to laboratory investigations and needs further development of genetic therapy. Nevertheless, it is a potent tool for precise pre-clinical investigations of JAK-STAT inhibition in cholangiocarcinoma. Thus, in the next chapter we focus on a detailed description of the main players of the JAK-STAT pathway in cholangiocarcinoma.

JAK-STAT SIGNALING UNDER CONDITIONS OF CHOLANGIOCARCINOMA PREDISPOSITION AND DEVELOPMENT

Presented data indicate that a number of receptors associated with JAK-STAT signaling are expressed in normal cholangiocytes, their expression increases under conditions of cholangiocarcinoma predisposition and is additionally elevated in cholangiocarcinoma cells (Table 1). Preferably used components of JAK-STAT signaling for such receptors are shown in Table 2. Thus, STAT5 and STAT3 proteins are the main downstream components for hormones, growth factors, and cytokines using JAK-STAT signaling in normal, proliferating, and malignant cholangiocytes and both STAT3 and STAT5 are implicated in promotion of cell survival in a variety of other normal and cancer tissues. SOCS3 is the primary negative regulator of cholangiocyte JAK-STAT signaling (Table 2). Among all these components, only STAT3 and SOCS3 have been investigated in cholangiocarcinoma cells.

STAT3

STAT3 plays an important role in transduction of survival signals downstream of both JAKs and receptor tyrosine kinases. Aberrant and frequently constitutive activation of STAT3 has been described in a variety of human cancers. Activation of STAT3 in tumors is associated also with tumor escape from immune attack[151,152].

Table 1 Cholangiocyte receptors associated with the JAK-STAT signaling pathways: alterations of expression during cholangiocarcinoma predisposition and development

Receptor type	Norma	Direction of alteration		References
		Cholangiocarcinoma predisposition	Cholangiocarcinoma development	
Prolactin receptor	Low	Up	Up Up	[7,58,148]
IL-6 receptor/gp130	Present	Up	Up Up	[2,5]
ErbB-2	Low/Absent	Up	Up Up	[2,138-140]
c-Met	Present	Up	Up Up	[2,5]
Growth hormone receptor	Moderate	Up	?	[72]
Leptin receptor	Present	?	?	[85]

Table 2 Known components of JAK-STAT signaling for receptors expressed in cholangiocytes

Receptor type	JAK	STATs	Negative regulators of JAK-STAT signaling	References
Prolactin receptor	JAK2	STAT5 > STAT3 > STAT1	SOCS3, SOCS1, SOCS2, CIS	[47,48,51-53]
Growth hormone receptor	JAK2	STAT5 > STAT3 > STAT1	SOCS3, SOCS1, SOCS2, CIS	[70,71]
Leptin receptor	JAK2	STAT3 > STAT5, STAT6, STAT1	SOCS3	[74-79]
IL-6 receptor/gp130	JAK1, 2, Tyk2	STAT3 > STAT1	SOCS3	[15,18-20,101,102,110-112]
ErbB-2	No, JAK3, TYK2	STAT3 > STAT5, STAT6	SOCS3, SOCS1, SOCS4, SOCS5	[23,132-136,149,150]
c-Met	No	STAT3 > STAT5, STAT1	SOCS3	[115-122]

In normal liver phosphorylated STAT3 localizes in epithelial cells lining large bile ducts and peribiliary glands[104]. STAT3 was constitutively phosphorylated in tested cholangiocarcinoma cell lines but not in nonmalignant cholangiocytes. Mcl-1 promotor has STAT3 binding site. STAT3 site-directed mutagenesis decreases Mcl-1 promotor activity[111].

SOCS3

STATs activation is negatively regulated by SOCS proteins. SOCSs are generally considered as a kind of tumor suppressor gene. In some hepatocarcinoma cell lines aberrant methylation in STAT-binding sites of the SOCS3 gene has been associated with loss of SOCS3 function and enhancement of tumor cell growth and migration[153]. Epigenetic inactivation of tumor suppressor genes by hypermethylation is relevant to cholangiocarcinoma development[154]. Known sustained STAT3 activation during cholangiocarcinoma progression may be partly due to SOCS3 epigenetic silencing due to hypermethylation of its promoter in human cholangiocarcinoma tissues and cell lines. Inverse correlation has been observed between phosphorylated STAT3 and SOCS3 protein expression in cholangiocarcinoma specimens. In cholangiocarcinoma samples failing to express SOCS3, extensive methylation of SOCS3 promoter has been shown in comparison with paired nontumor tissue. Methylation of SOCS3 promoter is also identified in two cholangiocarcinoma cell lines. Treatment with demethylating agent leads to restoration of SOCS3 expression, subsequent termination of STAT activation, and reduction of cellular levels of Mcl-1[112].

Cholangiocarcinoma markers and STAT-responsible genes

Based on the data on molecular alterations in human cholangiocarcinoma reviewed by Sirica, 2005, we have tried to analyze whether cholangiocarcinoma marker genes have putative STAT response elements in their promoters. As shown in Table 3 a number of genes involved in cholangiocarcinoma growth signaling, cell cycle regulation, and apoptosis inhibition possess candidate STAT3-, STAT5-, and STAT-1-binding sites in their promoters. Prl and IL-6 induced STAT5 and STAT3 activation is essential for up-regulation of their transcription activity in different tumor types and leads mainly to tumor progression[2,16,86,107,110-112,155,156].

We can assume that cholangiocarcinoma markers (presented in Table 3) including growth factors, members of pro-inflammatory signaling pathway, CDK inhibitors, cyclins, members of the antiapoptotic Bcl-2 protein family including Mcl-1, Bcl-2, Bcl-XL, may serve as final potential molecular targets of JAK-STAT cholangiocarcinoma signaling induced by Prl, IL-6, HGF, EGF and some other molecules acting *via* JAK-STAT signaling. These promoters STAT-binding sites may be involved in overexpression of these markers in cholangiocarcinoma cells.

It is important to note that down-regulation of CDK inhibitors ($p21^{waf1/cip1}$, $p27^{kip1}$) is a characteristic feature of cholangiocarcinoma development while activated STATs up-regulate them in some other tissues (Table 3), thus the role of JAK-STAT signaling cannot be restricted to progression cholangiocarcinoma.

PRIMARY NUCLEAR JAK-STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

Internalization pathways have also been demonstrated for signaling molecules associated with the JAK-STAT pathway. Nuclear accumulation of such molecules, ligand dependent nuclear translocation of corresponding receptors, nuclear localization of JAKs, and primary nuclear activation of STAT proteins have been revealed in different cell types and experimental systems. While

Table 3 Cholangiocarcinoma marker genes with promoter putative STAT responsible elements

Cholangiocarcinoma altered genes[1]	Direction of alteration[1]	Putative STAT responsible elements	Regulation by STAT	References
HGF	Up	STAT3	Up	[113,114]
VEGF	Up	STAT3	Up	[157]
COX-2	Up	STAT3, STAT5	Up	[158]
Cyclin D1	Up	STAT5	Up	[16,155]
p53	Down and up	STAT1	Up	[159]
$p21^{waf1/cip1}$	Down	STAT1, STAT3, STAT5	Up	[155,160,161]
$p27^{kip1}$	Down	STAT3	Up	[162]
Bcl-2	Up	STAT3	Up	[16]
Bcl-Xl	Up	STAT3, STAT5	Up	[155,163]
Mcl-1	Up	STAT3	Up	[111,112]
MUC1	Up	STAT3, STAT1	Up	[164]
MUC4	Up	STAT	Up	[165]

[1]Altered genes and direction of changes of their expression are adopted from[2].

membrane JAK-STAT signaling is the main signaling pathway in normal conditions, the importance of the primary nuclear pathway of JAK-STAT signaling rises under conditions of cell proliferation including preneoplastic and neoplastic proliferation. Appearance of components of JAK-STAT signaling in the nucleus or the enrichment of their nuclear pool seems to be associated with their participation in the regulation of proliferative processes and may be related to cholangiocarcinoma progression.

Ligands (such as Prl, GH, leptin, IL-1, IL-5, IFN-γ, TGF-α) and receptors acting *via* the JAK-STAT pathway or associated with STAT signaling by other mechanisms have been found in the nuclear compartment of different cell types mainly under conditions of high proliferative status and cancer development[8,10,166-169].

Nuclear translocation of Prl bound to Prl receptors has been revealed after Prl stimulation of Nb2 Prl-dependent lymphoma cells and the human breast cancer cell line T47D. Another mechanism suggests that Prl enters the nucleus in complex with cyclophilin B. In the nucleus this complex interacts with STAT5 and potentiates its activity facilitating STAT5 DNA binding. Prl/cyclophilin B complex directly interacts with the STAT5 N-terminus and appears to induce dissociation of PIAS3 from its complex with STAT5[170,171].

Both GH and GH receptor are subject to rapid nuclear translocation. Rat growth hormone binding protein (GHBP) that is a product of alternative splicing of GH receptor gene with an intact extracellular domain and without transmembrane and intracellular domains replaced by short carboxyterminal sequences, is also localized in cell nuclei in *in vitro* and *in vivo* experiments upon GH stimulation. Nuclear localized GHBP acts as a potent enhancer of STAT5 mediated transcription presumably by binding to PIAS proteins and thereby enhances the action of both GH and other members of the cytokine receptor superfamily[9,172].

Receptor tyrosine kinases associated with the JAK-STAT pathway or STAT signaling have also been found in the nuclear compartment. Nuclear EGF receptor (ErbB1) has been detected in highly proliferative tissues, such as regenerating liver, specimens of primary tumor, and cancer cell lines. Expression of nuclear ErbB1 correlates positively with increased level of cyclin D1 and negatively with overall survival in breast cancer patients. Nuclear ErbB1 co-localizes and interacts with importins α1β1, carriers that are critical for macromolecule nuclear import[8,168,169,173-176]. ErbB1 might act as a transcription factor to activate genes required for high proliferating activity. After EGF stimulation and ErbB1 nuclear translocation EGF-ErbB1 complex associates with cyclin D1 promoter element designated AT-rich sequence (RTRS)[177].

The ability of nucleus translocation is also inherent for other receptors of the ErbB family (ErbB2, ErbB3, and ErbB4). Rat p185neu, ErbB2, and ErbB3 receptors exist in the nucleus as full length molecules, while ErbB4 may undergo γ-secretase-mediated cleavage and with nuclear translocation of C-terminal 80-kDa fragment that represents a soluble cytoplasmic domain with an active tyrosine kinase region[10,178]. Nuclear ErbB2 associates with a specific nucleotide sequence (named HER-2-associated sequence - HAS) of the COX-2 gene promoter and is able to activate its transcription[168,173,175-178].

The hypothesis of a primary nuclear JAK-STAT signaling pathway is supported by data on constitutive expression of JAK1 and JAK2 in the cell nucleus. JAK1 and JAK2 molecules have been shown to contain nuclear localization sequences and both kinases have been revealed in nuclei of rat hepatocytes, pancreatic islet cells, CHO and some other cell types both without activation by upstream signaling and in active phosphorylated forms. GH treatment of cells stimulates phosphorylation of nuclear JAK2 without apparent changes of its subcellular localization. Active nuclear JAK2 phosphorylates transcription factor - nuclear factor 1-C2 (NF1-C2) in mammary epithelial cells[8,179,180]. Since both JAK1 and JAK2 are expressed constitutively in the nucleus and a number of cytokine receptors undergo nuclear translocation the realization of primary nuclear JAK-STAT signaling pathway seems very possible.

Primary nuclear STAT activation has also been observed. Nuclear ErbB1 has been shown recently to interact with STAT3 in the nucleus leading to transcriptional activation of inducible iNOS gene in breast carcinoma cells and this pathway may be associated with a high malignancy of other types of tumor cells with high level of nuclear ErbB1 and

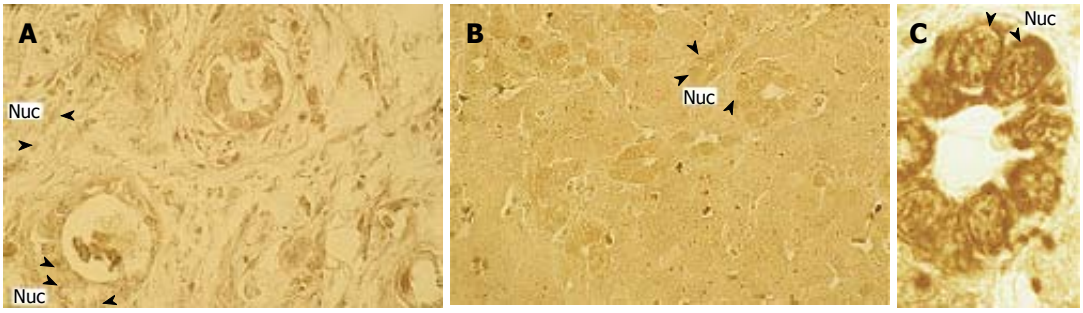

Figure 3 Immunohistochemistry for prolactin receptor (PrlR) expression in human cholangiocarcinoma sample (**A**) and in female (**B**) and male (**C**) rat liver tissue 14 d after common bile duct ligation. Intensive nuclear (Nuc), PrlR-positive immunoreactivity is shown. Orig. mag, **A**, **B**: x 40; **C:** x 100.

Table 4 Nuclear prolactin receptor expression in rat liver transplanted RS-1 cholangiocarcinoma cells and adjacent hepatocytes as compared with normal cells

	Nuclear PrlR-positive immunoreactivity (arbitrary units), medians (upper and lower quartiles)			
	Normal cholangiocytes	RS-1 cholangiocarcinoma cells	Normal hepatocytes	Tumor adjacent hepatocytes
Males	47 (0; 104)	191[b] (119; 288)	40 (26; 90)	308[b] (227; 381)
Females	113 (47; 194)	408[b] (256; 555)	59 (41; 101)	415[b] (266; 581)

[b]$P < 0.001$ as compared with correspondent intact group, Mann-Witney *U*-test.

STAT3[10]. C-terminal 80-kDa fragment of ErbB4 with nuclear traffic activity may associate with STAT5a in the nucleus and transactivate gene expression[178,181]. Intracellular ErbB4 domain possesses a nuclear localization sequence essential for its nuclear accumulation. In cotransfection experiments nuclear translocation of intracellular ErbB4 domain has been shown to be required for nuclear translocation of STAT5a and ErbB4 function as a nuclear chaperone for STAT5a transcription factor as has been suggested[182].

Thus, primary nuclear JAK-STAT signaling may become important at certain stages of the cell cycle or be critical for some populations of rapidly dividing or neoplastic cells.

Is a direct nuclear JAK-STAT pathway related to cholangiocarcinoma development? Intensive nuclear Prl receptor accumulation has been revealed in our laboratory in rat bile duct cells only after normal cholangiocyte transition to active proliferation during postnatal ontogenesis or after common bile duct ligation and is accompanied by elevation of total receptor content both in males and females[7]. Similarly, we have found nuclear localized Prl receptors with sharp elevation of their expression in samples of human cholangiocarcinoma tissues (Figure 3) as well as in liver transplanted rat cholangiocarcinoma cell line RS-1.

Both total content and nuclear expression of Prl receptors in transplanted RS-1 rat cholangiocarcinoma cells show moderate sex dependence with female predominance (Table 4). The intrahepatic RS1 cholangiocarcinoma transplant induces nuclear accumulation of Prl receptors not only in malignant cholangiocytes but also in adjacent and distant hepatocytes and increases total Prl receptor immunoreactivity in these cells[7,58].

We were unable to reveal prominent nuclear Prl receptor expression in hepatoma H27 cells after intrahepatic transplantation. Thus, nuclear accumulation of Prl receptors as well as their overexpression in intrahepatically transplanted RS1 cholangiocarcinoma cells and in hepatocytes of tumor bearing animals can serve as a specific marker of cholangiocarcinoma. Since we have used monoclonal antibodies U5 to the extracellular domain of rat Prl receptor, it was revealed that nuclear accumulation may be due to both full length Prl receptor or Prl binding protein as have been found for GH binding protein[9,172]. Appearance of Prl receptors in the nucleus is associated with Prl participation in the regulation of the proliferation process. This is confirmed by the data indicating that in Prl-dependent Nb2 lymphoma cells the cell cycle progression stops at the early G1 phase in the absence of Prl. Treatment of such cells with Prl leads to its transport to the nucleus followed by cell cycle progression[8,183].

In this respect unusual nuclear localization of prostaglandin E2 receptor EP1 that belongs to the G-protein coupled receptor superfamily detected in human cholangiocarcinoma cell lines seems very interesting[109].

Presented data show that primary nuclear JAK-STAT signaling may be relevant to cholangiocarcinoma progression and unusual nuclear localization of components of the JAK-STAT cascade may be considered as a predisposition to cholangiocarcinoma development.

CONCLUSION

While the role of GH, Prl and interleukins in normal cholangiocytes and pathophysiological states has been studied during the last decade, to date, the role of the JAK-STAT pathway in cholangiocarcinoma progression has not really been investigated and its importance is underestimated. Nevertheless, the summarized data provide a generalized view of the implication of the central cytokine signaling in tumor development of

epithelial bile duct cells. A more detailed study of JAK-STAT involvement in cholangiocyte proliferative diseases can open the door for newer therapeutic strategies serving a real alternative to surgery.

Moreover, an engagement of the new model of cancer (cholangiocarcinoma) to studies of JAK-STAT signaling can give us an exhaustive understanding of its role in cancer development at different stages, which is not yet completely clarified (promotive role in neoplasia development[33] and inhibitory role in metastasis progression[45]). Moreover, it can help us to decipher new mechanisms of activation of the components of the JAK-STAT pathway like receptor and/or ligand nuclear localization.

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