Oncogenic role of the Notch pathway in primary liver cancer (Review)

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Abstract. Primary liver cancer, which includes hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and fibrolamellar HCC, is one of the most common malignancies and the third leading cause of cancer-associated mortality, worldwide. Despite the development of novel therapies, the prognosis of liver cancer patients remains extremely poor. Thus, investigation of the genetic background and molecular mechanisms underlying the development and progression of this disease has gained significant attention. The Notch signaling pathway is a crucial determinant of cell fate during development and disease in several organs. In the liver, Notch signaling is involved in biliary tree development and tubulogenesis, and is also significant in the development of HCC and ICC. These findings suggest that the modulation of Notch pathway activity may have therapeutic relevance. The present review summarizes Notch signaling during HCC and ICC development and discusses the findings of recent studies regarding Notch expression, which reveal novel insights into its function in liver cancer progression.

Contents

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1. Introduction

Worldwide primary liver cancer, which includes primary hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and fibrolamellar HCC, is the fifth most common cancer and the third leading cause of cancer-associated mortality (1). Currently, surgery and liver transplantation are considered the optimal curative treatments for the disease (2); however, there is a significant shortage of organ donors, and surgical complications, recurrence and metastasis are common (3). Although epidemiological risk factors, including hepatitis B virus (HBV) and hepatitis C virus infection, have been identified, the molecular mechanisms underlying primary liver cancer remain unclear (4). Therefore, elucidation of the molecular pathogenesis of liver cancer and the development of effective targeted therapies is urgently required (5).

The Notch signaling pathway is evolutionarily conserved and controls numerous developmental processes, such as cell fate determination, terminal differentiation and proliferation (6). During embryonic development and adulthood, intracellular Notch signaling is required for cell specification, lineage commitment and maintenance of progenitor cells (7), in particular in the control of endothelial cell differentiation, arteriovenous specification and vascular development (8).

In mammals, the canonical Notch pathway includes four receptors (Notch1, 2, 3 and 4) and two ligand families [Jagged (JAG) 1 and 2 and Delta-like-ligand (Dll) 1, 3 and 4]. The Notch signaling pathway consists of ligand-induced activation of receptors, proteolytic cleavage and subsequent translocation of the notch intracellular domain (NICD) to the nucleus, where it functions as a transcriptional regulator (9). Activation of Notch signaling requires direct or indirect contact between cells expressing Notch ligands or receptors, and transmitting and receiving cells are subsequently modified by the interaction. Initially, cells express Notch receptors and ligands, and as the interaction continues, one cell becomes a transmitting cell by upregulating the expression of ligands and downregulating of receptors, while the receiving cells follow the opposite pattern (10). Prior to the NICD being transported to the nucleus, it is cleaved by the γ-secretase complex. In the nucleus, NICD interacts with C-repeat/DRE binding factor 1, a DNA-binding transcriptional repressor also known as the recombination

signal binding protein for immunoglobulin Kappa J region (RBPJ), and converts it into a transcriptional activator that induces the transcription of target genes, including the family of Hes and Hey-associated transcription factors. Furthermore, in the liver, Notch partially controls the expression of Sox9, HNF1 Homeobox B and transforming growth factor-β, which are key regulators in hepatic lineage commitment (11,12) (Fig. 1).

RBPJ is a DNA-binding protein, also known as CSL, which is a member of the Suppressor of Hairless (*Drosophila melanogaster*) family of transcription factors that recognizes the consensus sequence C(T)GTGGGAA. RBPJ predominantly acts as a transcriptional repressor of promoters that possess RBPJ binding sites via the recruitment of other co-repressors. The most important function of RBPJ is to mediate signals from Notch receptors. RBPJ is a common downstream transcription factor of Notch receptors and its absence indicates a complete block of the Notch signaling pathway (13).

The present review discusses the findings of recent studies regarding Notch expression and summarizes Notch signaling during HCC and ICC development.

2. Mutation of Notch-associated genes

Notch is a highly regulated signaling mechanism with numerous specific features. In humans, mutations in Notch ligands or receptors are associated with various diseases; for example, JAG1 and Notch2 mutations are associated with Alagille syndrome (14), and Notch3 mutations with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (15). Human genetic diseases and mutant mouse models have demonstrated the importance of Notch signaling in the development and remodeling of intrahepatic bile ducts (IHBD). Alagille syndrome (AGS) is a human autosomal dominant disorder that is caused by mutations in the Notch ligand JAG1, and less commonly in the Notch2 receptor (16). The estimated prevalence of AGS is 1 case per 70,000 live births worldwide (17). The disease is a multi-organ disorder most commonly diagnosed by liver abnormalities, which lead to hepatic bile duct paucity and cholestasis at birth (18). Cardiac, skeletal and ophthalmological abnormalities, and less frequently renal or vascular deficiencies, are also observed in patients with AGS (19). Numerous renal abnormalities are observed in patients with AGS, including renovascular disease, renal tubular acidosis, tubulointerstitial nephritis and renal dysplasia/hypoplasia (20). Notably, mice with haploinsufficiency for JAG1 exhibit no significant phenotypic abnormalities, suggesting that additional modifier genes contribute to the AGS phenotype observed in humans (21). Additionally, mutations in Notch3 are associated with inherited vascular diseases, including degenerative vascular disorder, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (22).

3. Notch pathway function in liver disease

The Notch signaling pathway is significantly associated with liver disease (23). In liver cirrhosis, the expression of Notch and Toll-like receptor signaling pathways are disordered (24). Based on the analysis of Notch and its target genes in HCC, the expression of Notch3 and Notch4 is aberrantly increased when compared with para-cancer tissue (25). Notch3 and 4 are rarely observed in normal liver tissue or para-carcinoma chronic hepatitis. In cirrhotic liver tissues, Notch3 mRNA and Hes6 mRNA expression levels are lower compared with those observed in HCC tissues. By contrast, high expression of Notch3 and low expression of Notch4 have been observed in human HCC HepG2 cell lines (26). Compared with HCC tissues of neoplastic cells, non-cancerous tissues adjacent to tumors have a high expression of Notch1 in the cytoplasm and Notch4 in nucleus, and a low expression of Notch2; however there is no significant difference between Notch4 and Notch3 expression. Thus, Notch receptor expression is abnormal in HCC (27). Rodent models with Notch loss or gain of function have demonstrated that Notch is involved in several stages of IHBD morphogenesis (28). Murine knockout (KO) studies for each of the mammalian Notch receptors and ligands have been conducted, and the resulting effects on the liver phenotype are presented in Table I (23-33).

Notch receptors

Overview. A number of Notch receptors have been identified in mammals, including Notch1-4. The receptors are transmembrane proteins with three domains: Extracellular Notch domain, a transmembrane domain and NICD. A number of studies have demonstrated that inhibition of the Notch signaling pathway induces the downregulation of Notch receptors (34,35).

Notch1. Notch1 regulates arteriovenous differentiation during embryogenesis and in the hepatic endothelium of adult mice (29). Homozygous disruption of the Notch1 gene is fatal at embryonic day (ED) 10, suggesting that Notch1 is essential for normal embryonic development. After ED10, histological analyses revealed widespread cell death, which was attributed to disorganized and delayed somitogenesis (30,31). Notch1 is required for vascular homeostasis of hepatic sinusoids by inducing quiescence and differentiation of liver sinusoidal endothelial cells. Thus, disruption of the Notch1 pathway leads to intussusceptive angiogenesis and nodular regenerative hyperplasia (36).

Notch2. Homozygous Notch2-deficient embryos exhibit developmental retardation, widespread cell death and embryonic mortality prior to ED11.5; however, normal somitogenesis is observed compared with a Notch1 KO (37). Alagille syndrome is also associated with Notch2 mutations (38). Jeliazkova *et al* (39) demonstrated that mice with a perinatal, liver-specific complete elimination of Notch2 exhibited a marked reduction in the number of mature bile ducts, an increased number of disorganized primitive biliary-like structures, portal inflammation, portal tract enlargement and fibrosis and biliary necrosis. Furthermore, neonatal Notch2 KO mice are severely jaundiced, with livers that exhibit no cytokeratin 19 positive ductal structures (40).

Notch3 and Notch4. Young Notch3 KO mice are viable and fertile without any apparent phenotypic abnormalities, whereas adult Notch3 KO mice exhibit arterial defects due to abnormalities in differentiation (41). In the liver, Notch3 regulates the activation of hematopoietic stem cells and may exhibit an anti-fibrogenic effect (42). Notch4 KO mice are viable and fertile, since during development Notch4 expression is restricted to vascular endothelial cells (42). However, Notch1/4 double KO mice exhibit a more severe phenotype,

Table I. Notch pathway phenotypes involved in liver development and regeneration in mouse models.

Author, year	Disrupted gene	Phenotype	(Ref.)
Croquelois *et al*, 2005 and Dill *et al*, 2012	Notch1	Nodular regenerative hyperplasia, disruption of homeostasis of hepatic sinusoids and stimulation of pre-and postnatal bile duct proliferation	(23) (24)
Geisler *et al*, 2008	Notch2	Impaired intrahepatic bile duct development	(25)
Chen *et al*, 2012	Notch3	Regulation of HSC activation. Interruption of Notch3 may be an anti-fibrotic strategy in hepatic fibrosis	(26)
Krebs *et al*, 2000	Notch4	Severe defects in angiogenic vascular remodeling	(27)
Hofmann *et al*, 2010	JAG1	Exhibition of Alagille syndrome, characterized by a paucity of intrahepatic bile ducts	(28)
Jiang *et al*, 1998	JAG2	Perinatal death	(29)
Redeker *et al*, 2013	Dll1	Stimulation of neuronal differentiation, lethal at embryonic day 11.5, severe somite patterning defects, hyperplastic CNS	(30)
Turnpenny *et al*, 2003	Dll3	Severe abnormalities in somitogenesis and recessive skeletal abnormalities in spondylocostal dysosotosis	(32)
Gale *et al*, 2004 and Djokovic *et al*, 2010	Dll4	Arteriovenous shunting, severe vascular remodeling defects	(33) (31)

JAG, Jagged; Dll, Delta-like-ligand; HSC, hematopoietic stem cells; CNS, central nervous system.

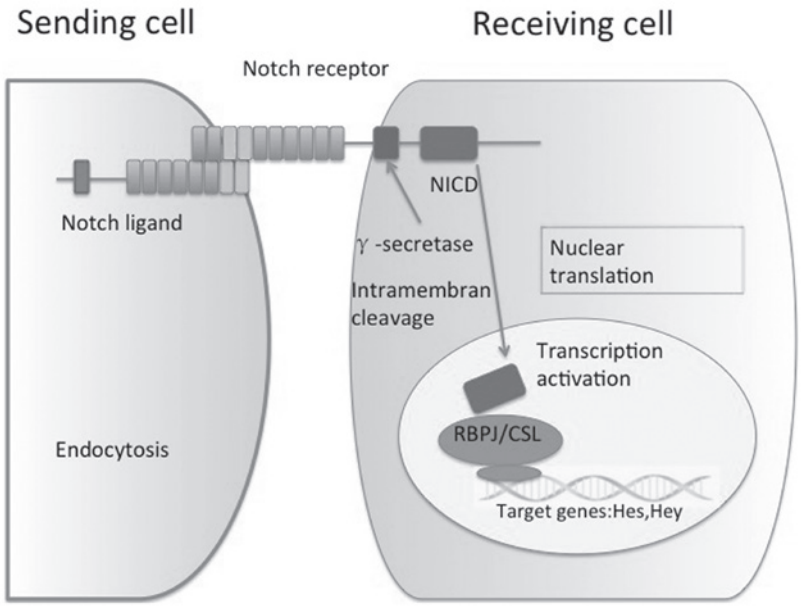

Figure 1. Model of the Notch pathway in major signaling cascades, including signal initiation through binding of the Notch ligand, cleavage of Notch, nuclear translation and transcriptional activation. NICD, notch intracellular domain; RBPJ, signal binding protein for immunoglobulin Kappa J region.

presenting with extensive defects in angiogenic vascular remodeling during embryonic development compared with Notch1 KO mice (43).

Notch ligands. Notch ligands include JAG1, JAG2, Dll1, Dll3 and Dll4. Homozygous disruption of Notch ligands invariably affects the liver. A previous study has demonstrated that deletion of JAG1 in the portal vein mesenchyme lead in jaundice, liver failure and small numbers of IHBDs (28). Furthermore, JAG2 KO mice die perinatally due to craniofacial defects, including fusion of the tongue with the palatal shelves, and syndactyly of the fore and hind limbs (29). Homozygous inactivation of Dll1 causes severe defects in somite patterning and the development of a hyperplastic central nervous system (44). Following ED9, Dll1 KO mice become hemorrhagic and die around ED11.5 (45). Dll3 is expressed in the presomitic mesoderm and is localized to the rostral somatic compartments. Homozygous disruption of Notch1 and Dll3 leads to severe abnormalities in somitogenesis. Mutations in the human Dll3 homolog result in recessive skeletal abnormalities in spondylocostal dysostosis (46). Dll4 is essential for embryonic vascular development and arterial specification and is clearly upregulated in the tumor vessels of humans and mice; Dll4 deficiency leads to severe vascular remodeling defects and embryonic mortality (31).

Notch transcription factor RBPJ. Human and murine RBPJ genes are located on chromosome 4 and 5, respectively (47) Canonical Notch signaling results in the upregulation of transcription via Notch target genes (48). RBPJ is a potent DNA-binding transcription factor that associates with a large number of chromatin regulators, corepressors and coactivators, and mediates Notch signaling (49). In mammals, RBPJ activates transcription by forming a ternary complex with one of the four Notch paralogs (Notch1-4) and the Mastermind (MAM) family of coactivators (MAMl1-3). NICD is released from the membrane and localizes to the nucleus, where it forms a transcriptionally active complex with RBPJ and the coactivator MAM. Assembly of the RBPJ-NICD-MAM ternary complex at a target gene acts as the switch for upregulating transcription (50).

Structural studies of Notch transcription complexes have identified the overall folds, domain organization and interacting regions of RBPJ, NICD and MAM proteins (51-53). RBPJ is composed of three domains: An N-terminal domain (NTD), a β-trefoil domain (BTD) and a C-terminal domain (CTD). The NICD binds RBPJ via a RBPJ-associated molecule and ankyrin (ANK) repeat domains that interact with BTD and CTD. MAM forms a helix with a distinctive bend, in which its N-terminal helical region forms a tripartite complex with ANK and CTD, and its C-terminal helical region binds the NTD of RBPJ. RBPJ binds the consensus DNA sequence, CGTGGGAA with moderate affinity (~200 nm Kd) (54,55), which is a similar site to that of the enhancer and promoter elements of Notch target genes (56). The structures of RBPJ and RBPJ-NICD MAM activator complexes, including assembling at various target genes, have enabled detailed biochemical and cellular studies. These transcriptionally active ternary complexes bind to the promoter and enhancer elements of Notch responsive genes, including hey1 and hes1 promoters (57) (Fig. 2).

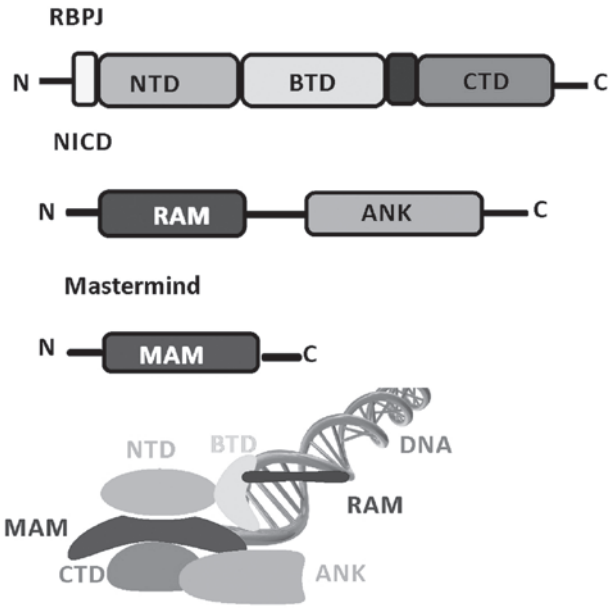

Figure 2. Schematic of the RBPJ structure and domains demonstrating the domain organization of RBPJ, NICD and MAM. The transcriptionally active RBPJ-NICD-MAM ternary complex binds to DNA. MAM is red and DNA is green. NTD, N-terminal domain; BTD, β-trefoil domain; CTD, C-terminal domain; RAM, RBPJ-associated module; ANK, ankyrin; MAM, Mastermind; RBPJ, signal binding protein for immunoglobulin Kappa J region; NICD, notch intracellular domain.

4. Role of Notch in HCC and ICC development

Liver cirrhosis is commonly observed during the early stages of HCC and ICC (58). Previously, alterations in the Notch pathway have been identified in various solid tumors, such as breast, ovarian, pancreatic and liver cancer, melanoma and glioblastoma (59). The Notch signaling pathway exhibits a carcinogenic role in HCC. However, various members of the Notch signaling pathway may function as suppressors or enhancers of HCC. For example, the suppressive effect of Runt-related transcription factor 3 in HCC may be a result of Notch signaling inhibition (60). The Notch signaling pathway is involved in stem cell self-renewal and differentiation, and Notch signaling has been reported to promote the self-renewal of cancer stem-like cell niches in primary and metastatic tumors. Notch activation triggers epithelial-mesenchymal transformation (61). Several studies have revealed that a loss of the epithelial phenotype via epithelial-mesenchymal-transition promotes the acquisition of a stem-like phenotype and drug resistance (62-64). In addition, in patients with HCC an epithelial gene signature has been associated with sensitivity to the epidermal growth factor receptor inhibitors gefitinib and cetuximab (65). Deregulated expression of Notch, including the overexpression and activation of Notch proteins, ligands and targets, has been identified in numerous solid tumors, including cervical, endometrial, renal, lung and gastric carcinomas (66). These findings suggest that additional signaling pathways may affect whether Notch functions as a tumor suppressor or oncogene in a particular tissue (67).

Gain or loss of function mutations in the Notch pathway have been identified in several types of cancers, including neural stem cell tumors, lung carcinomas and prostate cancer (68). Although Notch does not directly lead to unregulated cell proliferation or genetic alterations that are associated with tumor progression, it alters the developmental state of cells and consequently maintains cells in a proliferative or undifferentiated state (69). In chronic HBV infection (CHB), repression of Notch receptors was demonstrated to lead to immune dysfunction (70). The contribution that a decreased expression of Notch receptors makes to ongoing fibrosis, cirrhosis and HCC is unclear; however, repression of Notch receptors in CHB has been suggested to repress immune regulation, resulting in the inhibition of differentiation and proliferation of effector cells leading to additional pathogenesis of CHB (71). Notably, the pro-mitogenic function of Notch was demonstrated in a model of partial hepatectomy (72). The pro-oncogenic function of Notch was also investigated by genome wide analysis of HCC samples, which revealed that the Notch coactivator MAML2 is a target of genetic alterations (73). Additionally, Notch signaling is crucial for the differentiation of hepatocytes into biliary lineage cells during the early stages of ICC developments. Gain and loss of function studies have demonstrated that ICC develops via the Notch-mediated differentiation of hepatocytes into biliary lineage cells, and that the malignancy and progression of the tumor are dependent on the intensity of Notch signaling in hepatocytes (74). Notably, hepatitis-infected

Table II. Various liver cancer phenotypes associated with the Notch pathway.

Author, year	Mouse model	Liver defects	Liver tumorigenesis	(Ref.)
Dou *et al*, 2008	Dll4 KO	Inhibition of hepatic vascular alterations	Inhibition of tumor growth	(9)
Zong *et al*, 2009	AFP, AlbCre/RBPJ, NICD	Reduction in bile duct number	Association with ICC	(12)
Dill *et al*, 2012	Mx1cre/RBPJflox	Disruption of vascular homeostasis	Spontaneous angioma	(24)
Djokovic *et al*, 2010	Dll4 KO	Inhibition of hepatic vascular alterations	Inhibition of tumor growth	(31)
Fan B *et al*, 2012	AlbCre/Hes1, Notch1flox	ICC	ICC arises through Notch-mediated hepatocytes	(85)
Villanueva *et al*, 2012	AFP Cre/NICD KO	Dysplasia	Differentiated HCC	(84)
Viatour *et al*, 2011	AdCre/TKO	Stem/progenitor cell expansion	HCC development	(86)
Vincent *et al*, 2009	Hu-AGT-TG	Reduced liver volume, blood flow velocities and arterial vessel density	Antagonized tumor angiogenesis and delayed tumor growth	(87)

Dll, Delta-like-ligand; KO, knockout; AFP, α-fetoprotein; RBPJ, signal binding protein for immunoglobulin Kappa J region; NICD, notch intracellular domain; ICC, intrahepatic cholangiocarcinoma; TKO, technical knockout; HCC, hepatocellular carcinoma.

hepatocytes may be converted into biliary lineage cells via Notch signal activation and thus become the point of origin for ICC (75). Therefore, suppression of Notch signaling may present a novel strategy for the treatment of ICC, since it may inhibit the conversion of hepatocytes into biliary lineage cells during the early stages of ICC development (76). Mice with liver-specific constitutive activation of Notch1 intracellular domain (N1ICD) may develop HCC when they reach adult age (77). Genomic profiling technology has revealed that a Notch-specific gene expression signature reported in mice overexpressing NICD was also present in a cluster of patients with HCC (78). Histological analyses of the mouse liver tissue exhibited similar features compared with that of the HCC patients, which included the presence of proliferating K-19 positive cells. Constitutive Notch2 overexpression causes HPCs to spontaneously develop into dedifferentiated HCC cells (79). In addition, Notch-induced malignant hepatocyte transformation is associated with downregulation of hepatocyte-associated genes and Sox9 expression (80). Fate-mapping studies have demonstrated that clear-cell adenocarcinoma (CCA) cells derived from hepatocytes may be converted to a biliary K-19 positive phenotype (81). Accordingly, in CCA development, N1ICD association with protein kinase B signaling in hepatocytes stimulated their malignant dedifferentiation (82). The stimulation of N1ICD expression by inflammatory mediators has also been reported in human CCA, which additionally supports the role of Notch in liver cancer (83) (Table II) (9,12,24,31,84-87).

5. Notch-based therapeutic strategies in liver cancer

Persistent activation of Notch signaling may lead to oncogenesis depending on modifier factors, including the inflammatory environment or the presence of other carcinogenic conditions that may cause HCC or CCA (88). A Notch signature has been reported in a subset of HCC patients and an overexpression of Notch receptors has been reported in human CCAs, which is fundamentally required for the development of targeted therapies (89). Silencing of the Notch pathway may potentially inhibit Notch-driven tumor progression and interfere with tumor aggressiveness, since Notch activation has been associated with a more malignant phenotype (84). However, the identification of a reliable tissue-specific biomarker of Notch inhibition is critical for the application of Notch-targeted therapy. Notably, the hepatic Notch target gene Sox9 is associated with a poor prognosis in liver cancers (90). Therefore, the role of Sox9 as a potential biomarker of Notch involvement and indication for Notch-targeted treatment requires additional study.

Roma *et al* (91) reported that pharmacodynamically active drugs, including emtricitabine/rilpivirine/tenofovir or 'GSI', inhibits Notch signaling, which prevents metastasis and recurrence of tumors and increases the disease-free survival time of patients. However, gastrointestinal toxicity is a primary side-effect of GSI use and these drugs are more effective against tumors that exhibit upregulated Notch signaling. Therefore, other potential modulating factors remain to be identified (92).

6. Conclusion

An increased understanding of the mechanisms involved in liver cancer proliferation and differentiation may aid the development of therapeutic strategies for liver cancer. The Notch pathway is emerging as a critical signaling pathway, which regulates cell proliferation, differentiation and necrosis associated with normal development, as well as stem cell renewal and differentiation. In conclusion, loss or disruption of Notch signaling may be a key contributing factor in bile ductular disorders, sinusoidal capillarization and the neovascularization of portal regions in the liver. To develop effective therapeutic approaches for the treatment of liver cancer, additional studies that investigate the Notch pathway are urgently required.

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