Notch signaling in the pathogenesis, progression and identification of potential targets for cholangiocarcinoma (Review)

PEERANATE VANAROJ[1], WANNA CHAIJAROENKUL[1] and KESARA NA-BANGCHANG[1,2]

[1]Graduate Program in Bioclinical Sciences, Chulabhorn International College of Medicine, Thammasat University; [2]Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma, Thammasat University, Pathumthani, 12120 Thailand

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Abstract. Cholangiocarcinoma (CCA) is an aggressive type of bile duct cancer that is characterized by a high mortality rate due to its late diagnosis and ineffective treatment. The aim of the present systematic review was to analyze the association between Notch signaling and CCA in terms of its pathogenesis, progression and potential treatment targets. Relevant information was gathered from the PubMed, ScienceDirect and Scopus databases using the search terms 'cholangiocarcinoma' AND 'Notch signaling'. Of the 90 articles identified, 28 fulfilled the eligibility criteria and were included in the analysis. It was concluded that overexpression/upregulation of Notch ligands, such as Jagged1 and Notch receptors (Notch1, Notch2 and Notch3), as well as upregulation of the upstream Notch signaling pathway, promoted CCA development and progression. In addition, downregulation of Notch1 signaling through several possible interventions appears to be a promising strategy for inhibition of CCA development and progression. Therefore, the Notch signaling pathway may be considered as a potential target for CCA control.

Introduction

Cholangiocarcinoma (CCA) is a bile duct cancer with a high mortality rate. A significant proportion of the CCA cases and resultant mortalities worldwide are reported in the northeastern region of Thailand, where the major risk factor is infection by the liver fluke *Opisthorchis viverrini* through the consumption of improperly cooked cyprinoid fish that contain the parasite (1). There are currently no specific biomarkers for early detection of early-stage CCA, and patients are usually diagnosed when the disease has already progressed to the advanced stage, resulting in a poor prognosis. Liver resection is the standard therapy, but is not suitable for all cases. The 5-year survival rate following liver resection is <20%, depending on the aggressiveness, metastatic propensity and invasiveness of the tumors. Furthermore, CCA is resistant to chemotherapy and radiation (2). Investigation of the molecular mechanisms underlying the pathogenesis and progression of CCA has been an ongoing research focus, and several signaling molecules and pathways have been demonstrated to be involved in the pathogenesis and progression of CCA (3).

The Notch signaling pathway has been proposed as a conservative pathway that plays a key role in cell differentiation, proliferation and apoptosis (4). This signaling pathway is associated with several receptors and ligands. The four identified receptors are Notch1, Notch2, Notch3 and Notch4. The two families of ligands involved are the Delta-like family (DLL1, DLL3 and DLL4), and the Jagged family (Jagged1 and Jagged2). The activation of Notch signaling relies on two proteolytic enzymes in the a disintegrin and metalloprotease (ADAM) families and γ-secretase enzyme, which cleaves Notch receptors into two domains, i.e., Notch extracellular domain (NECD) and Notch intracellular domain (NICD). Following cleavage, NICD translocates to the nucleus and binds to transcription factors to promote expression of target genes, such as members of the hairy and enhancer of split (Hes) and Hes-related to YRPW motif (Hey) families (5).

Overexpression of Notch signaling genes is associated with the proliferation of certain cancers, including ovarian and breast cancers and glioma (6-8). However, overexpression of Notch signaling genes can also result in cancer cell apoptosis, such as in cases of liver cancer, small cell lung cancer and melanoma (9-11). The Notch signaling pathway in CCA cells is summarized in Fig. 1. The objectives of the present systematic review were to analyze the association of Notch signaling with the pathogenesis and progression of CCA, and uncover potential molecular targets for CCA control.

Materials and methods

The present systematic review was performed by combining the search results from three databases, i.e., PubMed, ScienceDirect and Scopus. The search terms applied were

Correspondence to: Dr Kesara Na-Bangchang, Graduate Program in Bioclinical Sciences, Chulabhorn International College of Medicine, Thammasat University, 99 Moo 18, Paholyothin Road, Klonglung, Pathumthani, 12120 Thailand
E-mail: kesaratmu@yahoo.com

Key words: cholangiocarcinoma, Notch signaling, Notch1, Notch2, Notch3

'cholangiocarcinoma' AND 'Notch signaling'. All articles were retrieved and downloaded to the EndNote X9 database (Thomson Reuters Company, Canada) for further analysis. They were initially screened by titles and abstracts to exclude irrelevant articles (those not involving CCA or the Notch signaling pathway). Full-text articles included after the initial screening were further evaluated by applying the predefined eligibility criteria. The inclusion criteria were as follows: i) Articles published between January 2004 and March 2020; ii) articles available as full-text articles in English; and iii) articles with *in vitro/in vivo/ex vivo* studies related to the Notch signaling pathway in CCA alone or CCA and hepatocellular carcinoma (HCC). The exclusion criteria were as follows: i) Articles related to other diseases or types of cancer; ii) articles related to pathways other than Notch signaling; iii) duplicated articles; or iv) review articles, letters to the editor, editorials, systematic analyses or meta-analyses.

Two reviewers extracted data independently and disparities were resolved by discussion and suggestions from the third reviewer. The information extracted for analysis included: First author's name and year of publication, objective(s) of the study, type of Notch receptor investigated, type of study (*in vitro, in vivo* and *ex vivo*), type of cell lines or animals used, laboratory techniques used, and key results and conclusions.

Results

A total of 89 articles from PubMed, ScienceDirect and Scopus databases were downloaded to the EndNote database. A total of 54 articles were excluded, and further analysis of the titles and abstracts of the remaining 36 articles led to the exclusion of 8 articles (5 articles unrelated to CCA, and 3 articles unrelated to the Notch signaling pathway in CCA). Finally, 27 articles were included in the analysis. The flow diagram of the study selection process is presented in Fig. 2, and the study summary is provided in Tables I and II. The associations of Notch1 and Notch2 signaling with CCA development and progression were investigated in 6 articles each, while those of Notch3 and Notch4 signaling were investigated in 3 and 1 article(s), respectively. The investigations involved *in vitro* (n=8), *in vivo* (n=12) and *ex vivo* (n=8) studies. The effects of modulators of Notch signaling as potential chemotherapeutic targets for CCA were investigated in 10, 5, 3 and 3 articles for Notch1, Notch2, Notch3 and Notch4 signaling pathways, respectively. The investigated modulators included cinobufagin, verteporfin-photodynamic therapy (PDT), γ-secretase inhibitor (GSI), γ-secretase inhibitor IX, endocannabinoids, corilagin, short hairpin (sh)RNA, and small-molecule inhibitors (SMIs) of aspartate-β-hydroxylase, anti-Notch1,2,3 and Jagged1, microRNA (miRNA)-34a, PIK3-catalytic subunit alpha (PIK3CA), PIK75, verteporfin, FLI-06, microfibrillar-associated protein 5 (MFAP5), ALW-II-41-27, ephrin A1, lymphotoxin β receptor (LTβR), xanthohumol and γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Table II).

Discussion

A summary of the currently available information on the association between Notch signaling and the pathogenesis and progression of CCA, including modulators (inhibitors or stimulators) of the signaling pathway as potential candidates for CCA chemotherapeutics, is presented in Fig. 3.

Upregulation of Notch signaling has been proposed as the mechanism associated with the transformation of mature hepatocytes into CCA cells (12-14). Nevertheless, activation of Notch signaling in hepatic progenitor cells, but not the transformation of hepatocytes, is proposed as the mechanism underlying CCA development (15). Increased expression of Notch1 has been linked to CCA development and progression (14,16-24). Notch1 has also been associated with cyclin E, the coordinate regulator protein in the G1 phase of the cell cycle; additionally, cyclin E can induce DNA damage (15). Increased NICD1 expression has also been associated with CCA development and progression through upregulation of cyclin E-associated DNA damage (15,25). Furthermore, Notch1 and Notch2 signaling have been reported to play a critical role in CCA formation (12-14,24,26-29), in which Jagged1 is the specific ligand. Upregulation of PIK3CA, AKT, and Jagged1 directly activates Notch 2 signaling and induces CCA development. Overexpression of Jagged1 enhances Notch2 signaling (26), while anti-Jagged1 treatment suppresses Notch2 signaling (27,28,30,31). At this time, the information on Notch3 and its role in CCA development and progression arelimited (24,32). Notch4 signaling has also been reported to promote the development of intrahepatic CCA (ICC) and is associated with a poor survival rate (24).

The activation of upstream Notch signaling molecules, including Yes-associated protein (YAP), AKT, mTOR, SNAIL and PIK3CA, are key processes that stimulate CCA formation through the transformation of mature hepatocytes into CCA cells (12). AKT is the main upstream Notch signaling molecule, which upregulates Notch1 and Notch2 (12-14,26,27,29,33). mTOR is another upstream activator of the Notch1 receptor (12,16). YAP is an upstream signaling molecule for both AKT and mTOR (12), and co-expression of YAP with AKT induces CCA development through activating Notch signaling via the Notch2 receptor and Jagged1 ligand (12,14,28,34), or with co-expression of NICD and shp53 (shRNA of p53) (35). Co-expression of AKT and Ras (the protein product of the oncogene KRAS2), on the other hand, induces tumorigenesis (21,27,29). MFAP5, enhanced green fluorescent protein-positive cells, mTOR and AKT, activate Notch signaling by increasing Notch1 expression, thereby enhancing CCA cell proliferation (12,16,17). Modulation of mutant genes, such as p53 (inactivation), isocitrate dehydrogenase1 (activation), or other pathways, such as the Wnt (β-catenin) pathway (activation), and the Myc pathway (activation) with co-expression of Notch signaling (activation) has been reported to induce CCA development (14,25,33,36). Upregulation of Notch1 expression may also be caused by additional factors, such as a high expression level of presenilin 1 (37). Aspartate β-hydroxylase (ASPH) enhances activation of Notch signaling and stimulates CCA cell proliferation and migration. A high expression level of Notch1 can activate Ras-related C3 botulinum toxin substrate 1, which promotes CCA cell invasion and migration. ephrin type-A receptor 2 enhances the expression level of Notch1 and promotes CCA growth through the activation of AKT/RAS and by promoting lymphatic metastasis in ICC (21).

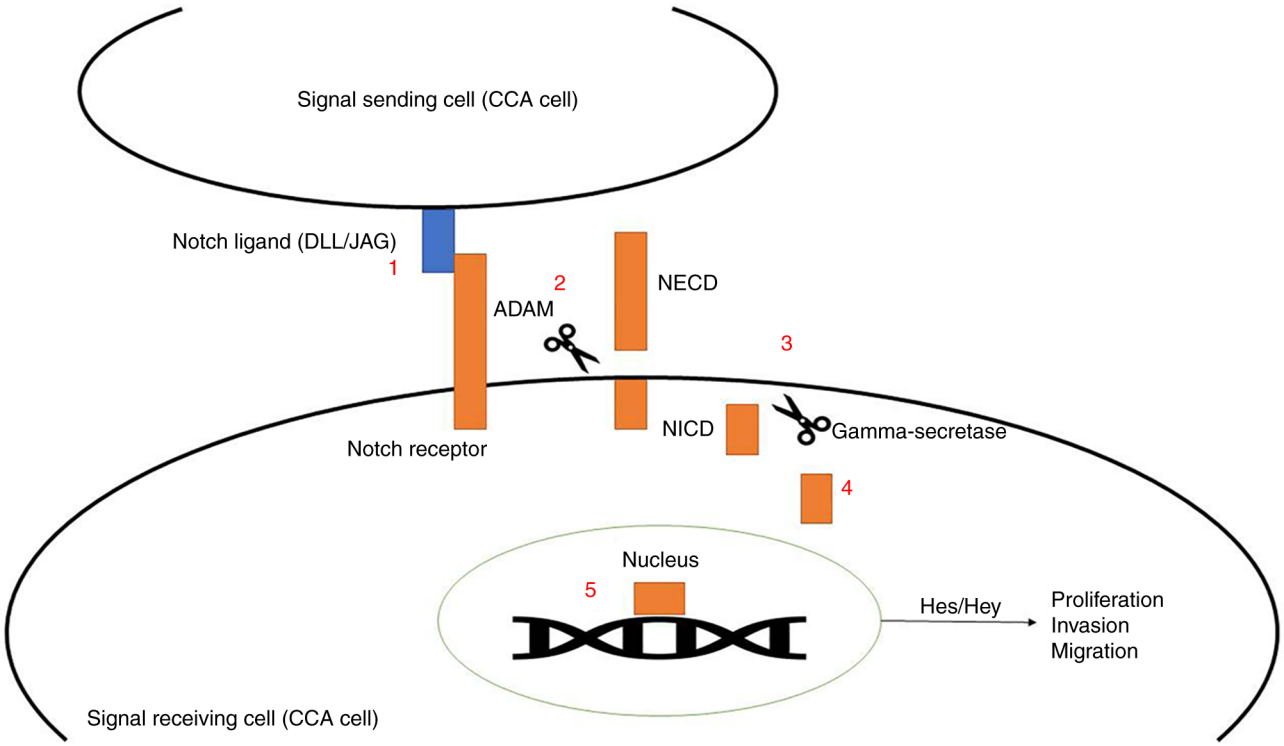

Figure 1. Notch signaling pathway in CCA cells. Notch ligands, such as members of the DLL or the JAG family, attach to the Notch receptors to activate the ADAM and γ-secretase enzymes. The ADAM cleaves NECD outside the cell membrane and the γ-secretase enzyme cleaves the NICD inside of the cell membrane. After being cleaved, the NICD translocates to the nucleus and interacts with the DNA-binding protein to promote expression of the target genes, such as Hes and Hey, which are associated with proliferation, migration and, invasion of CCA cells. CCA, cholangiocarcinoma; Hes, hairy and enhancer of split; Hey, Hes-related to YRPW motif; ADAM, a disintegrin and metalloprotease; NECD, Notch extracellular domain; NICD, Notch intracellular domain; DLL, delta-like family; JAG, Jagged family.

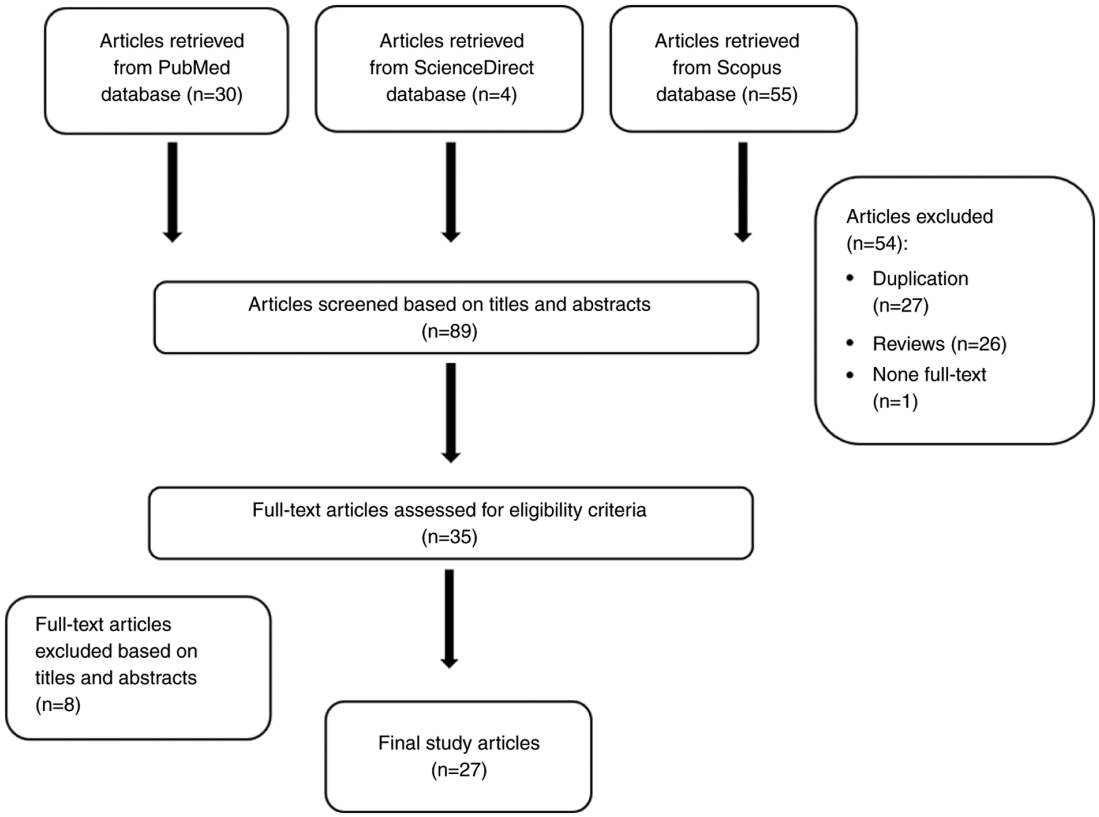

Figure 2. Flow diagram of study identification and selection process. A total of 90 articles were selected by the title and abstract by using the key words 'cholangiocarcinoma' AND 'Notch signaling.' A total of 54 articles were excluded (duplication, review articles, or non-full-text articles). A total of 8 articles were further excluded by the titles and abstracts, and 24 articles were finally included in the analysis.

Table I. Studies related to Notch signaling and pathogenesis and progression of CCA.

Author (year)	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Ishii *et al* (2010)	Role of AFP-producing cells as cancer stem cells	Notch1	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) HuCCT-1, OZ, SSP-25, RBE ii) NOD/SCID mice iii) Human ICC samples	Flow cytometry, single-cell culture assay, cell proliferation assay, anchorage-independent cell growth assay, H&E staining, sphere formation assay, RT-PCR, immunohistochemistry, histology	i) EGFP-positive cells: AFP upregulating Notch1 activation through NICD; increasing Notch 1 production. ii) DAPT: Inhibiting activation of Notch1 signaling; reducing number of EGFP-positive cells. iii) AFP-producing cells: Cancer-initiating cells.	(16)
Che *et al* (2016)	Role of Jagged1 in ICC	Notch2	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) HUCCT-1, KKU-156 ii) FVB/N mice iii) Human ICC samples	Immunohistochemistry, RT-qPCR, western blotting, vimentin staining, Picro-Sirius Red staining, histology	Jagged1 co-expression and AKT activation: Increasing Notch2 expression and Hes1 proteins; inducing ICC development/ progression.	(27)
Ding *et al* (2016)	Oncogenic potential of IDH1R132C (IDH1 mutant gene) in ICC development	Notch1	*In vivo*	FVB/N mice	Immunohistochemistry, Picro-Sirius red staining, histology	Combination of IDH1R132C, shP53 and NICD1: Stimulating ICC development independent of AKT/mTOR and Ras/MAPK signaling.	(25)
Guest *et al* (2016)	Notch3 signaling in CCA using thioacetamide driven model	Notch3	i) *In vivo* ii) *Ex vivo*	i) Rat, CK19CreYFPp53$^{f/f}$ mice ii) Human CCA samples, non-cancerous liver samples	PCR array, RT-qPCR, western blotting, immunohistochemistry, immunofluorescence, MTT assay, histology	i) Overexpression of Notch3: CCA formation/progression through inhibiting RBPJ, co-transcription factor with Notch signaling ii) Activation of Notch3: CCA development, inhibited by shRNA.	(32)
Zhang *et al* (2018)	Role of Hippo pathway (Salvador-Warts-Hippo pathway) in AKT/Ras-driven hepatocarcinogenesis	Notch2	i) *In vitro* ii) *In vivo*	i) SNU-475, SK-HEP-1 ii) FVB/N mice	Histology, immunohistochemistry, western blotting, RT-qPCR, colony formation assay	i) Hippo pathway: Inactivated in AKT/Ras liver tumors, leading to Yap/TAZ nuclear localization ii) Activation of the Hippo pathway or blocking of Yap/TAZ transcriptional activity: -AKT/Ras-induced tumor development: Delayed ICC-like lesion formation: Blocked	(28)

Table I. Continued.

Author (year)	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
						i) Hippo pathway: Notch2 expression downregulation ii) Overexpression of Lats2 or dnTEAD2: Inhibiting HCC growth; decreasing ICC-like markers and Notch2 expression	
O'Rourke *et al* (2020)	Anti-CCA activity of Pan-GSI; transcriptomics of the Notch receptors and network analysis of major signaling pathways in CCA; Notch receptor engagement, γ-secretase modulation as a therapeutic approach	Notch1, Notch3	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) HuCCT-1, SNU-1079, SSP-25, RBE, SNU-308, YSCCC, SNU-869, SNU-245, SNU-1196, KMCH, WITT, SNU-478, KMBC ii) Mice iii) Human CCA tissues, surrounding liver tissues	Immunohistochemistry, RT-PCR, western blotting, Immunofluorescence, RNA-seq analysis, DNA methylation, CCA tissue microarray, histology	i) Notch1: Significantly increased; imbalancing Notch pathway. ii) GSI cocktail: Effective anti-CCA	(23)
Yamamoto *et al* (2017)	Interactions of the PI3K-AKT, YAP and Myc pathways in liver tumorigenesis and phenotypic determination	Notch1, Notch2	i) *In vivo* ii) *Ex vivo*	i) C57BL/6J mice ii) Human HCC samples, human ICC sampless	RT-qPCR, microscopy, immunohistochemistry, histology, Picro-Sirius red staining	i) PI3K-AKT pathway activation: Key determinant of cell differentiation ii) AKT activation by Myc and Notch1/2: Inducing high-grade CCA iii) AKT and YAP activation: Inducing low-grade CCA iv) AKT/Myc: Inducing low-grade CCA v) Co-activation of AKT, Myc and YAP: Inducing high-grade HCC vi) Co-activation of YAP and Myc: Inducing hepatoblast/stem-like cells	(12)

Table I. Continued.

Author (year)	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Xu *et al* (2019)	Role of SNAIL in promoting EMT and metastasis in hepatocarcinogenesis	Notch2	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) HLE, SNU449 ii) FVB/N mice iii) Human HCC tissues, non-tumorous surrounding liver tissues	Macroscopy, histology, immunohistochemistry, Picro-Sirius red staining, immunofluorescence, western blotting	i) Overexpression of SNAIL: Not associated with metastasis, but associated with CCA formation ii) SNAIL: CCA-like phenotype regulation in hepatocarcinogenesis *via* regulation of Yap and Notch. iii) SNAIL mRNA level: Sstrongly correlated with CCA marker expression	(29)
Tschaharganeh *et al* (2014)	Role of p53 in regulating Nestin in HCC and CCA	Not specific Notch	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) Hep-3B, HuH-7, HepG-2 ii) Nestin promoter-GFP mice, FVB/N mice, p53 fl/fl mice, Alfp-Cre p53 fl/fl mice iii) Human HCC tissues, human HCC-CCA tissues, human CCA tissues	Immunohistochemistry, RT-PCR, histology, micro array, DNA sequencing, immunofluorescence, western blotting, luciferase assay, chromatin immunoprecipitation, co-immunoprecipitation, colony formation assay, microscopy	i) p53: Regulating expression of stem and progenitor cell-associated protein nestin (required for tumor initiation) ii) p53: Loss/mutation in the majority of human cancers; facilitating mature hepatocyte differentiation into nestin-positive progenitor-like cells (differentiate into HCC or CCA cells response to lineage-specific mutations targeting Wnt and Notch signaling)	(34)
Wang *et al* (2018)	Role of Notch signaling and Notch receptors in AKT/YAP-driven ICC formation	Notch2, Notch1	i) *In vitro* ii) *In vivo*	i) KKU-M-213, RBE, HuCCT-1, HLE, SNU-449, SNU-475 ii) R26-EYFP mice, FVB/N mice, FVB/N and Notch2$^{flox/flox}$ mice, FVB/N and Notch1$^{flox/flox}$ mice	Immunofluorescence, western blotting, immunohistochemistry, histology, RT-qPCR	i) AKT/YAP: Activating Notch cascade; inducing hepatocyte-derived ICC; decreasing tumor proliferation (with Notch signaling inhibition) ii) Ablation of Notch1: Delaying ICC tumorigenesis iii) Notch2: Essential for AKT/YAP-induced ICC development: Suppressing Notch2: Reducing tumor proliferation, Notch1 expression, SOX9-positive cell, JNK pathway; delaying ICC tumorigenesis	(13)

Table I. Continued.

Author (year)	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
						iv) Notch2 deletion inactivation: Switching tumor phenotype from ICC to hepatocellular adenoma-like lesions v) Notch1 inactivation of Notch1 in hepatocytes: No significant histo- morphological changes	
Zhou *et al* (2013)	Expression and role of Notch1 in cell migration in ICC	Notch1	i) *In vitro* ii) *In vivo*	i) Human ICC tissues, human non-cancerous tissues ii) QBC939, RBE, ICC-9810	RT-PCR, western blotting, bromodeoxyuridine incorporation analysis, Rac activation assay, immunocytochemistry, migration assay, microscopy, immunofluorescence, immunohistochemistry	i) High expression of Notch1: ICC tissues/cell lines ii) Notch1: Activating Rac1, promoting cell migration iii) Rac1: Tumor cell invasion and migration iv) GSI: Downregulating Notch1 Rac1; inhibiting cell migration	(17)
Wu *et al* (2014)	Expression of Notch receptors in ICC	Notch1, Notch2, Notch3, Notch4	*In vitro*	Human ICC tissues, human non-Cancerous tissues	Immunohistochemistry	i) Notch1 exhibited 82.9% immunostaining positivity: 51.2% low-grade immunoreactivity and 31.7% high-grade immunoreactivity ii) Notch2 exhibited 56.1% immunostaining positivity;: 26.8% low-grade immunoreactivity and 29.3% high-grade immunoreactivity iii) Notch3 exhibited 39% immunostaining positivity, 34.1% low-grade immunoreactivity and 4.9% high-grade immunoreactivity iv) Notch4 exhibited 34.1% immunostaining positivity: 24.4% low-grade immunoreactivity and 9.8% high-grade immunoreactivity	(24)

Table I. Continued.

Author (year)	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
						v) Notch1 and Notch4 were upregulated in ICC cells compared with non-tumor cells vi) Notch1 was expressed in tumors sized >5 cm vii) Notch4 was expressed in cases with serum carbohydrate antigen 125 >35 U/ml and was correlated with poor survival rate	

AFP, α-fetoprotein; CCA, cholangiocarcinoma; dnTEAD, double-negative TEA domain; EGFP, enhanced green fluorescent protein; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; ICC, intrahepatic CCA; IDH1, isocitrate dehydrogenase 1; LATs, large tumor suppressor; NICD, Notch intracellular domain; Pan-GSI, Pan γ-secretase inhibitor; PDAC, pancreatic ductal adenocarcinoma; PIK3CA, PIK3 catalytic subunit alpha; RT-qPCR, reverse transcription-quantitative PCR; RBPJ, recombination signal binding protein for immunoglobulin kappa J region; SNAIL, snail family transcriptional repressor 1; YAP, Yes-associated protein.

The role of Notch signaling in cancer pathogenesis and progression has also been demonstrated in several other types of cancer, including embryonal carcinoma (38), glioblastoma (39), melanoma (40), ependymoma (41), breast cancer (42), HCC (34), ovarian cancer (43), endometrial carcinoma (44), esophageal squamous cell carcinoma, gonadotroph pituitary adenomas (42), rhabdomyosarcoma (45), colon cancer (46), gastric cancer (47), gastrointestinal stromal tumors (48), anaplastic thyroid cancer (49), medullary thyroid cancer (50), pancreatic cancer (51), glioblastoma multiforme (52) and neuroendocrine neoplasms (53). The signaling molecules and pathways involved vary according to the type of cancer. Notch3, in addition to Notch1, appears to play an important role in breast cancer development and progression through its activation of cartilage oligomeric matrix protein expression (54).

Downregulation of Notch1 signaling by several interventions has been demonstrated to be a promising strategy for inhibition of CCA growth. These interventions include administration of cinobufagin (a traditional Chinese medicine extracted from parotid and skin glands of Chinese Toad) (21), xanthohumol (55), verteporfin-PDT (30), DAPT (23), PIK75 (PIK3CA-specific inhibitor), verteporfin, anti-Notch1 antibody (27), miRNA-34a (31) and small interfering (si)RNA LTβR (22). The downregulation of Notch1 siRNA expression reduces Notch1 levels, which results in inhibition of Notch signaling, suppression of cell proliferation, and promotion of apoptosis (20,22,27,30,31,55). Verteporfin-PDT downregulates the mRNA expression of Notch1, Notch2 and Jagged1 (30); additionally, verteporfin can reduce YAP levels, decrease cell proliferation and induce apoptosis (14). Inhibition of MFAP5 using the γ-secretase inhibitor FLI-06 also suppressed Notch1 expression in CCA (20). Inhibition of Notch2 signaling using anti-Notch2 or anti-Jagged1 antibodies also suppressed Notch2 signaling (27) and miRNA-34a expression (31). Direct inhibition of Notch2 using miRNA-34a, PDT, anti-Notch2 or anti-Jagged1, and the Hippo pathway cascade decreases Notch2 levels, promotes apoptosis and inhibits cell proliferation. However, Notch1 and Notch2 signaling have been demonstrated to interact antagonistically with each other (27,36). Antagonists of Notch1 signaling can enhance Notch2 signaling, while Notch2 depletion can increase the levels of various components of the Notch1 signaling pathway, such as the endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG), or anti-Notch1 and anti-Notch2 antibodies (27,34). AEA and 2-AG have been shown to exert different effects on Notch signaling. AEA, which has antiproliferative activity, upregulates Notch1 signaling via increasing the level of presenilin1, a catalytic subunit of γ-secretase. On the other hand, 2-AG, which has growth-promoting activity, upregulates Notch2 signaling via increasing the expression of presenilin 2, another catalytic subunit of γ-secretase. 2-AG activates Notch2 and enhances CCA cell proliferation (36). There have only been a limited number of studies related to Notch3 and its role in CCA, although is has been shown that using gene knockout or shRNA and SMIs to decrease Notch3 levels suppresses CCA growth (32). Both shRNA and SMIs inhibit ASPH and Notch signaling to suppress CCA cell proliferation and migration (56).

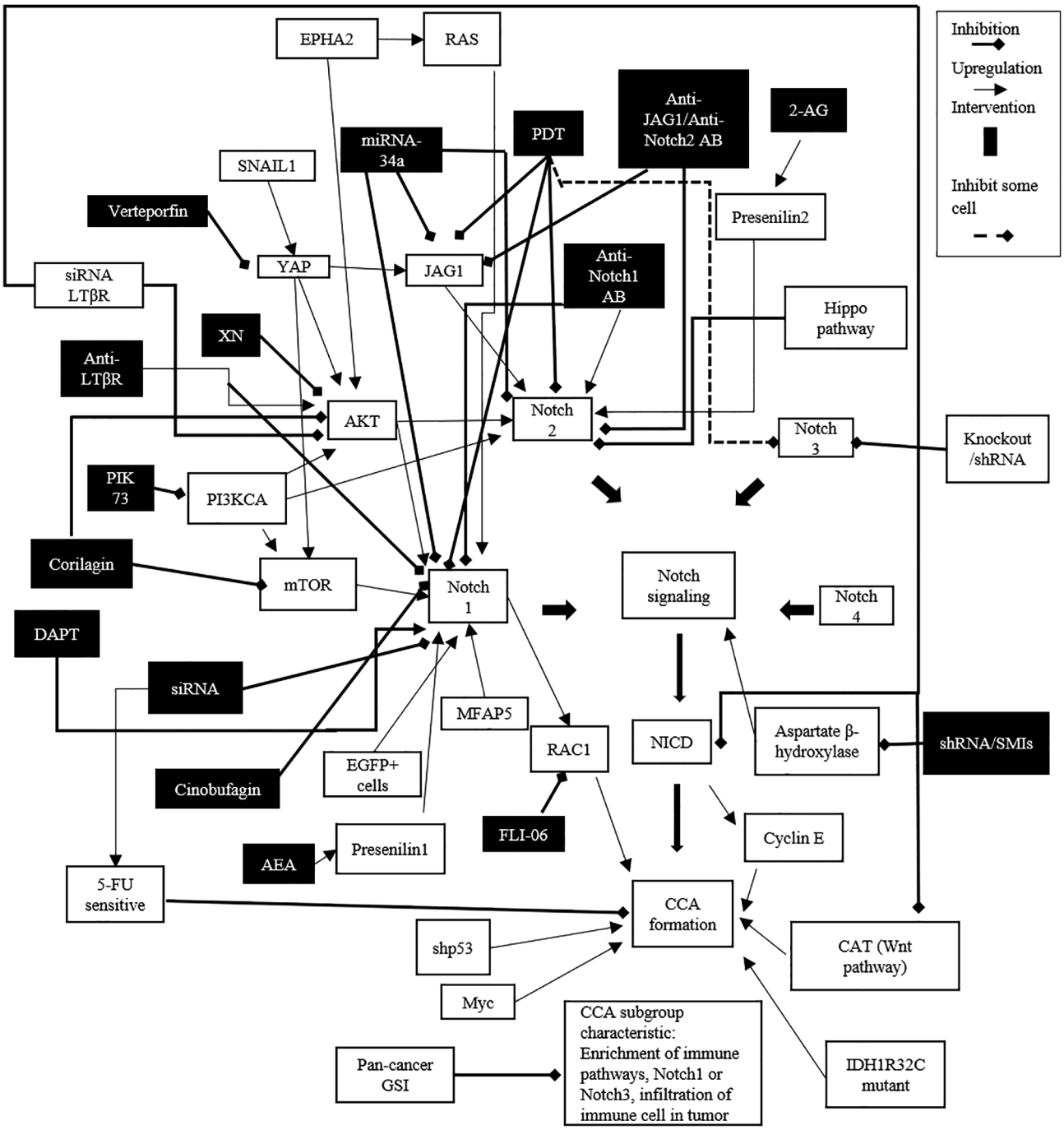

Figure 3. Notch signaling and ist association with the pathogenesis, progression and chemotherapeutic targets in CCA. Notch receptors, such as Notch1, Notch2, Notch3 and Notch4, activate the Notch signaling pathway and promote CCA formation via NICD. The upstream signals of Notch, such as mTOR, PI3KCA, AKT, YAP and SNAIL, can promote the effects of the Notch signaling pathway by increasing Notch receptor expression levels. A direct ligand of Notch2, such as JAG1, can also upregulate Notch2 expression and can be upregulated by YAP. Notch1 can be induced by MFAP5 and is found in EGFP$^+$ cells. Moreover, Notch1 can activate RAC1 to promote CCA formation, or induce NICD to increase cyclin E expression to promote CCA formation. Several interventions downregulate the expression of Notch receptors and suppress Notch signaling, including anti-LTβR, verteporfin, corilagin, XN, PDT, cinobufagin, PIK73, siRNA, shRNA/SMIs, DAPT, miRNA-34a and FLI-06. In addition, some modulators can inhibit certain Notch receptors but promote different Notch receptors, such as AEA, 2-AG, anti-Notch1 antibody, anti-Notch2 antibody and anti-JAG1 antibody. Some pathways, including the Hippo pathway, decrease Notch receptor expression levels, while others, such as the Wnt pathway, can promote CCA formation. CCA, cholangiocarcinoma; NICD, Notch intracellular domain; YAP, Yes-associated protein; PI3KCA, PIK3-catalytic subunit alpha; MFAP5, microfibrillar-associated protein 5; EGFP, enhanced green fluorescent protein-positive; RAC1, Ras-related C3 botulinum toxin substrate 1; LTβR, lymphotoxin β receptor; PDT, photodynamic therapy; siRNA, small inhibitory RNA; shRNA, short hairpin RNA; SMIs, small-molecule inhibitors; DAPT, γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; miRNA, microRNA; AEA, anandamide; 2-AG, 2-arachidonylglycerol; XN, xanthohumol.

The potential of the aforementioned interventions for the control of other types of cancer has also been demonstrated. These include cinobufagin for osteosarcoma (57), xanthohumol for hepatocarcinoma (58), FLI-06 for tongue cancer (59), GSI for glioblastoma cancer stem cells (60), T-cell for acute lymphoblastic leukemia (61), osteosarcoma (62) and

Table II. Studies on modulators of Notch signaling in CCA.

Author (year)	Modulators	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Ren *et al* (2019) (2019)	Cinobufagin (from the parotid and skin glands of the Chinese toad)	Anti-CCA activity	Notch1	i) *In vitro* ii) *In vivo*	i) QBC39, RBE ii) Nude mice	Cell counting assay, colony formation assay, flow cytometry, western blotting, histology	Inhibiting expression of Notch1, Hes1, Hes5, NICD; inducing CCA cell apoptosis; inhibiting tumor growth	(21)
Cerec *et al* (2009)	Verteporfin-PDT	Constitutive expression of the Notch signaling in CCA; anti-CCA activity of PDT and GSI; effect of verteporfin-PDT on Notch signaling	Notch1, Notch2, Notch3	*In vitro*	TFK-1, HuCCT-1	Cell proliferation assay, RT-qPCR	i) Decreasing mRNA levels of Notch1, Notch2, Jagged1, Notch3 in TFK-1 cell ii) Decreasing mRNA levels of Notch1, Notch2 and Jagged1; no effect on Notch3 mRNA in HuCCT-1	(30)
El Khatib *et al* (2013)	GSI	Anti-CCA activity of GSI IX	Not specific Notch	i) *In vitro* ii) *In vivo*	i) TFK-1, SZ-1, EGI-1 ii) RSA26 NICD mice	WST assay, western blotting, MTT assay, wound healing migration assay, Transwell assay, anchorage-independent cell growth assay, flow cytometry, immunohistochemistry, histology	i) Inhibiting cell migration, invasion, colony formation; inducing CCA apoptosis ii) Inducing CCA cell cycle arrest (SubG1); delaying cell division process iii) Overexpressing Notch signaling and inactivating p53: Stimulating CCA development	(35)
Frampton *et al* (2010)	Endocannabinoids (AEA, 2-AG)	i) Opposing effects of AEA and 2-AG in differential activation of Notch signaling ii) Differential activation of Notch signaling associated with different types of presenilin in γ-secretase-containing cells	Notch1, Notch2	i) *In vitro* ii) *In vivo*	i) Mz-ChA-1 ii) Balb/c nude mice	qPCR, western blotting, immunofluorescence, immunohistochemistry, MTS assay, bromodeoxyuridine staining, immunoprecipitation, TUNEL assay, PCNA, histology	i) AEA (antiproliferative activity): Increasing presenilin1 expression and activation of Notch1 signaling ii) 2-AG (growth-promoting activity) increasing presinilin2 expression and activation of Notch2 signaling iii) Notch1 signaling and Notch2 signaling: Antagonists of each other.	(36)

Table II. Continued.

Author (year)	Modulators	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Gu *et al* (2016)	Corilagin (from *Dimocarpus longan* Lour.)	Effect of corilagin on regulation of tumor development and occurrence through Notch signaling	Notch1	i) *In vitro* ii) *In vivo*	i) Mz-ChA-1, QBC-9939 ii) Old athymic nude mice	Flow cytometry, monolayer wound healing assay, RT-qPCR, western blotting, co-immunoprecipitation, histology	i) Inhibiting CCA cell proliferation, migration, invasion; promoting apoptosis (G2/M); inhibiting Notch1 and Notch signaling (Notch-mTOR, by reducing Hes1 mRNA through inhibiting Hes1 promoter activity) ii) Inhibiting CCA growth; repressing Notch1 and mTOR expression	(19)
Huang *et al* (2016)	shRNA, SMIs targeting ASPH	i) Molecular mechanism by which ASPH mediates CCA malignant phenotype ii) Potential of ASPH as CCA therapeutic target	Not specific Notch	i) *In vitro* ii) *In vivo*	i) ETK-1, H-1, NEC, RBE, SSP-25 ii) Nude mice, Fisher-344 male rats	Western blot analysis, RT-PCR, Transwell invasion assay, cell growth assay, MTT assay, colony formation in soft agar assay, cancer stem cell sphere formation assay, histology	i) shRNA and SMIs: Inhibiting ASPH activity; downregulating Notch signaling; inhibiting cell proliferation/migration; enhancing caspase-3 cleavage; inducing apoptosis; suppressing tumor growth/progression	(56)
Huntzicker *et al* (2015)	Anti-Notch1, anti-Notch2, anti-Notch3, anti-Jagged 1 antibodies	Role of Notch signaling when Notch receptors are physiologically activated via their ligands	Notch1, Notch2, Notch3	i) *In vitro* ii) *In vivo*	i) FVB/N mice ii) Human HCC samples	Immunofluorescence, RT-qPCR, histology, western blotting, immunohistochemistry, MRI, RNA-seq analysis	i) Notch1 inhibition: Reducing tumor burden (lowering large HCC-like tumors, but increasing CCA-like tumors) ii) Jagged1 acts as a ligand of Notch2: Inhibition of Notch2 and Jagged1: Reducing both HCC-like and CCA-like tumors iii) Notch3 inhibition: No significant effect on tumor burden	(26)

Table II. Continued.

Author (year)	Modulators	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Kwon *et al* (2017)	miRNA-34a	Biological function and epigenetic regulation of miRNA-34a in CCA	Notch1, Notch2	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) CCLP-1, SG-231, HUCCT-1, TFK-1, H-69 ii) SCID mice iii) Human CCA tissues, non-neoplastic peribiliary glands	Western blot analysis, RT-qPCR, cell proliferation assay, colony formation assay, bisulfide conversion PCR, methylation-Specific PCR, chromatin immunoprecipitation assay, immunohistochemistry, histology, cell growth assay	i) miRNA-34a: Decreasing Notch1, Notch2 and Jagged1 levels; inhibiting tumor growth/colony formation ii) EZH2-mediated H3K27 trimethylation and DNA methylation: Suppressing miRNA-34a expression independently iii) EZH2 methylation: At 3 points of H3k7 of histone iv) DNA methylation: Silencing at CpG islands on the promoter of miRNA-34a gene	(31)
Li *et al* (2015)	PIK3CA, YAP inhibitors (PIK75, verteporfin)	Concomitant activation of PI3K and YAP on CCA carcinogenesis	Notch2	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) HLF, SK/Hep-1, EGI-1 ii) FVB/N mice iii) Human HCC samples, human CCA samples, human mixed HCC/CCA samples	Histology, immunohistochemistry, western blotting, cell proliferation assay, cell death detection, ELISA	i) Co-expression of PIK3CA/YAP: Activating downstream pathways (AKT/mTOR, ERK/MAPK, Notch2 signaling); Rapid tumor development ii) PIK3CA and/or YAP inhibitors: Suppressing cell proliferation; inducing apoptosis	(14)
Li *et al* (2019)	FLI-06, MFAP5 (Notch inhibitor)	Expression and function of FLI-06, MFAP5 on ICC	Notch1	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) RBE, SSP-25 ii) Nude mice iii) Human ICC samples, human HCC samples, human healthy samples	Immunohistochemistry, RT-qPCR, ELISA, western blotting, co-immunoprecipitation, western blotting formation assay, RNA-seq, qPCR ATAC-seq, GSEA analysis	i) MFAP5: Upregulated in patients with ICC. ii) MFAP5: Promoting ICC cell proliferation (inhibiting p21, thus increasing CCND1/CDK4/6/CDC25A transcription, accelerating G0/G1 transition to S). iii) MFAP5: Directly bound to Notch1 receptor upregulating Notch1 signaling; increasing chromatin accessibility, thus ICC aggressiveness.	(20)

Table II. Continued.

Author (year)	Modulators	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Sheng *et al* (2019)	ALW-II-41-27, EphrinA1 (EPH receptor tyrosine kinase inhibitor)	Genetic alternations (EPHA2 mutation) during lymph node metastasis of ICC; potential mechanism and clinical strategy	Notch1	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) HUVEC, CCLP-1, HUCCT-1 ii) NOD/SCID mice iii) Human ICC samples, primary cells from ICC tissues	Whole exome seq, RNA-seq, western blotting, RT-PCR, Immunohistochemistry, ELISA, Transwell assay, PCR-based sanger seq, Cell proliferation assay, Pathway enrichment analysis, Transcriptome seq	i) EPHA2 mutant type D739N: activating Ser897 phosphorylation; promoting lymphatic metastasis via VEGF secretion (inducing tumor plasticity, lymphangiogenesis) ii) EPHA2 mutation: Enhancing AKT/RAS, activating Notch1 signaling, promoting lymphatic metastasis iii) EPHA2 mutation: Inhibited by ALW-II-41-27, but not ephrinA1 (major ligand of EPHA2).	(22)
Scarzello *et al* (2016)	Anti-LTβR	Relationship between LTβR and HCC, and ICC initiation	Not specific Notch	i) *In vitro* ii) *In vivo*	i) HepG-2, Huh-1, HLE, Huh-7, Oz, KMBC, HuCCT-1, Mz-ChA-1 ii) C57/BL6 Jax mice	Gaussia luciferase assay, immunohistochemistry, western blotting, qPCR, flow cytometry, microarray analysis, Oil red O stain, histology, transcriptomics	i) AKT/CAT: Initiating liver tumorigenesis; reducing tumor burden after LTβR expression ii) Anti-LTβR: Promoting tumor aggressiveness/progression; increasing AKT levels, thus upregulating NICD. iii) siRNA of LTβR: Decreasing AKT, NICD, CAT, Hes1 levels; reducing tumor progression	(33)
Walden *et al* (2017)	XN	Effect of XN on CCA	Notch1	i) *In vitro* ii) *In vivo*	i) CCLP-1, SG-231, CC-SW-1 ii) Athymic nude mice	MTT assay, colony formation assay, Flow cytometry, Western blot, Luminescence assay, Histology	i) Inducing cell cycle arrest; increasing p21 expression and inducing apoptosis and inhibiting cell proliferation and colony formation ii) Reducing Notch1 and AKT phosphorylation iii) Suppressing tumor growth/burden	(55)

Table II. Continued.

Author (year)	Modulators	Objective to investigate	Notch type	Study type	Cell/animal used	Techniques	Results and conclusions	Study (Refs.)
Wu *et al* (2014)	siRNA Notch1	Effect of Notch1 expression in ICC tissues/cells	Notch1	i) *In vitro* ii) *In vivo*	i) RBE, HCCC-9810 i) Human ICC tissues	qRT-PCR, Western blot, Cell counting assay, Colony formation assay, Transwell invasive assay, flow cytometry, DAPI staining	i) siRNA: Knocking down Notch1 activity and thus downregulating ICC proliferation/invasiveness; sensitizing ICC to 5-fluorouracil by suppressing ABCB-1 and MRP-1 expression ii) Notch1: oOverexpressed in ICC cell membranes and cytoplasm.	(18)
Zender *et al* (2013)	DAPT	Function of Notch signaling liver cancer formation	Notch1, Notch3	i) *In vitro* ii) *In vivo* iii) *Ex vivo*	i) Mz-ChA-1, TFK-1, Egl-1, Hep-3B, HepG-2 ii) Nude mice, ROSA26 NICD mice, AlbCre mice iii) Human CCA tissues	Immunohistochemistry, Histology, Feulgen staining, Flow cytometry, Western blot, RT-PCR, Luciferase assay, Caspase3/7 assay, TUNEL staining	i) NICD overexpression in hepatocytes: Inducing endoduplication cycle and severely impairing cell proliferation ii) NICD overexpression in hepatic progenitor cells: Inducing CCA *via* cyclin E, leading to genetic instability iii) CCA: Upregulating Notch1 and Notch3 (correlated with cyclin E level) iv) DAPT: Decreasing Notch1 and Notch3 level, downregulating cyclin E expression, inducing apoptosis and tumor remission	(15)

AEA, endocannabinoids anandamide; 2-AG, 2-arachidonylglycerol; ASPH, aspartate-β hydroxylase; ATAC-seq, Assay for Transposase-Accessible Chromatin with high-throughput sequencing; CCA, cholangiocarcinoma, DAPT, γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EZH2, enhancer of zeste homolog 2; GSEA, Gene Set Enrichment Analysis; GSI, γ-secretase inhibitor; HCC, hepatocellular carcinoma; LTβR, lymphotoxin β receptor; ICC, intrahepatic CCA; MFAP5, microfibrillar-associated protein 5; NICD, Notch intracellular domain; PDT, photodynamic therapy; PIK3CA, PIK3 catalytic subunit alpha; qPCR, quantitative PCR; RNA-seq, RNA-sequencing; YAP, Yes-associated protein; XN, xanthohumol; SMIs, small-molecule inhibitors; siRNA, small inhibitory RNA; shRNA, short hairpin RNA; miRNA, microRNA; Hes, hairy and enhancer of split.

triple-negative breast cancer (63), and DAPT for glioma (64), colorectal cancer (65), cervical cancer (66), gastric cancer (67), head/neck squamous cell carcinoma (68), osteosarcoma (69), choriocarcinoma (70) and ovarian cancer (71). In addition, miRNA-34a has also been reported to inhibit the progression of pancreatic cancer and medulloblastoma (72,73), and siRNA interference has been reported to inhibit cell proliferation in glioblastoma multiforme (74).

In summary, overexpression/upregulation of the expression of Notch ligands (e.g., Jagged1) and Notch receptors (Notch1, Notch2, Notch3 and Notch4), as well as upregulation of the expression of upstream Notch signaling molecules, promotes CCA development and progression. Therefore, downregulation of Notch1 signaling through several interventions is a promising strategy for inhibition of CCA development and progression. However, further studies focusing on the application of these modulators of Notch signaling in a clinical setting must be performed in the future.

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Authors' contributions

PV, WC and KN conceived the study and analyzed and interpreted the data. PN performed the background research, collected the data and wrote the manuscript. WC and KN critically revised the manuscript for important intellectual content. Data authentication is not applicable. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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