

Aberrant mRNA splicing generates oncogenic RNA isoforms and contributes to the development and progression of cholangiocarcinoma (Review)

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Abstract. Cholangiocarcinoma is a lethal biliary cancer, with an unclear molecular pathogenesis. Alternative splicing is a post-transcriptional modification that generates mature mRNAs, which are subsequently translated into proteins. Aberrant alternative splicing has been reported to serve a role in tumor initiation, maintenance and metastasis in several types of human cancer, including cholangiocarcinoma. In this review, the aberrant splicing of genes and the functional contributions of the spliced genes, in the carcinogenesis, progression and aggressiveness of cholangiocarcinoma are summarized. In addition, factors that influence this aberrant splicing that may be relevant as therapeutic targets or prognosis markers for cholangiocarcinoma are discussed.

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1. Introduction

Cholangiocarcinoma (CCA), is a malignant tumor that arises from the biliary epithelial tissue and is highly aggressive, with no effective pharmacological treatment available. This cancer has a poor prognosis and a high mortality rate (1). The highest worldwide incidence of CCA is found in the North and Northeast of Thailand, at ~85 cases per 100,000 individuals per year (2). The major predisposing factors for CCA in Asia are infection by the liver fluke, *Opisthorchis viverrini* (3,4) and exposure to groups of food-borne carcinogens, especially N-nitrosodimethylamine compounds identified in grilled or fermented foods (5). The only effective treatment for the disease is surgery. For patients who are not eligible for surgical therapy, gemcitabine- or 5-fluoro-uracil (FU)-based treatments are given. These are largely ineffective, since the response rate is only 20-30%.

The molecular pathology of bile duct cancer has been a topic of intense study. The molecular pathogenesis of CCA usually involves abnormal signal transduction and pro-inflammatory secretion, facilitated by gene mutations and epigenetic dysregulations (on a set of oncogenes and tumor suppressor genes) (6). Several lines of evidence also indicate that the abnormal expression of growth factors and receptors, the RAS/RAF/ dual specificity mitogen-activated protein kinase kinase 1 pathway, and the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin pathway may be involved with CCA initiation, maintenance, and metastasis (7). Several studies reported that specific-target drugs or inhibitors, including epithelial growth factor receptor (EGFR; Lapatinib or Erlotinib), fibroblast (F) GFR and PI3K inhibitor, (8) may be applicable to CCA. A number of novel therapeutics are under evaluation in a phase 2 study (9).

Alternative splicing (AS) is a post-transcription modulation process that can generate a variety of gene isoforms. Spliced mRNA is able to be translated to different amino acids with various biological functions (10). Pre-mRNA is spliced through the spliceosome; a large macromolecule comprising 5 small nuclear ribonucleoproteins (snRNPs U1, U2, U4/U6 and

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U5). The AS generates 5 common splicing patterns, including alternative 5' splice site, alternative 3' splice site, exon skipping, intron retention and mutually exclusive exons. Previous data demonstrates that aberrant alternative splicing also includes exonic regulatory element mutation, splice site mutation and altered splice isoform ratios. The differential expression of splicing factors is implicated in various diseases and linked to hallmarks of cancer (11-15). A number of reports demonstrated a correlation between aberrant AS and tumor initiation/progression (16-20). The truncated oncogenic forms of the proteins, resulted from aberrant AS involved in cancer cell growth, apoptosis, drug resistance and angiogenesis.

Aberrant splicing of macrophage-stimulating protein receptor (RON) (21) and Rac1 (22) promoted angiogenesis and epithelial mesenchymal transition (EMT) phenotypes. In addition, a BRAF (V600E) spliced isoform, lacking exon 4-8 induced vemurafenib drug resistance in melanoma (23). In the present review, evidence is presented that supports important roles for aberrant splicing and the spliced isoforms of the genes, in CCA carcinogenesis and cancer aggressiveness.

2. Relevance of aberrant AS in cholangiocarcinoma development, progression and aggressiveness of phenotypes

A number of articles have summarized the interconnection between AS and cancer progression, including 17 genes in lung cancer (16), 2 reports in breast cancer in which 7 genes (17) and 9 genes (18), respectively were demonstrated, and 9 genes in hepatocellular carcinoma (19,20). The global cancer-specific transcript variants of five cancers demonstrated protein metabolism and modification are the most prevalent functional processes in cancer (24). As mentioned previously, aberrant AS has been discovered and proven to have functional involvement in the initiation and progression of cancer. In CCA, 623 genes presented with alternative splicing in CCA samples when compared with healthy bile duct tissue samples (25). In this review, atypical splicing of nine genes, which have been investigated at the *in vitro*, *in vivo* and clinical levels, and their relevance to CCA pathogenicity are summarized. The structure of nine pre-mRNAs that undergo alternative mRNA splicing to generate wild-type mRNA or variant transcripts are presented in Fig. 1. The derived-spliced transcripts or protein isoforms are summarized by how they can facilitate various characteristics of a cancer cell, as presented in Fig. 2 and Table I.

Cluster of differentiation (CD)44v6 and CD44v8-10. CD44 is a transmembrane glycoprotein receptor that specifically binds to extracellular hyaluronan and other extracellular matrix (ECM) proteins to activate signal transduction, and serves important roles in tumor proliferation, migration, and invasion (26,27). CD44 pre-mRNA encodes transmembrane and cytoplasmic-tail regions. The AS of CD44 can generate up to 12 isoforms of proteins with different biological functions. The CD44v isoforms participate in cancer progression: CD44v6 promotes EMT and activates the transforming growth factor- β pathway (28,29), and CD44v8-10 is involved in poor cancer prognosis (30,31). Expression of CD44v6 can be linked to CCA proliferation. CD44v6 is a CCA-specific isoform that has never been detected in normal bile ducts (32). Furthermore,

the CD44v8-10 transcript was overexpressed in CCA and was demonstrated to stabilize the xCT system, a cysteine/glutamate transporter, to elevate glutathione synthesis and inhibit reactive oxygen species (ROS) accumulation in CCA cells. This function of CD44v8-10 was demonstrated to facilitate cancer cell survival in cases caused by liver fluke-induced inflammation. In addition, upregulation of CD44v8-10 suppressed p38 mitogen-activated protein kinase 1 (MAPK), which is a signaling protein involved in ROS suppression. Although the mechanism by which the CD44 spliced isoform may suppress p38 is still unclear, this observation appeared to be clinically relevant, since patients with CD44v overexpression and negative-phosphorylated (phospho)-p38MAPK have significantly shorter survival times compared with low CD44v expression and positive-phospho-p38(MAPK) (33).

WISP1v. Wnt-inducible secreted protein 1 [(WISP1) also known as CNN4] is a member of the cysteine-rich CCN family of proteins, which are highly expressed in skeletal tissues and has a role in bone formation and maintenance. Functions of this protein involve cell proliferation, osteoblastic differentiation and migration (34,35). WISP1 comprises 4 domains, including insulin-like growth factor-binding protein (IGFBP), VWC, thrombospondin-1 (TSP-1) and CT domains and is known to have variants with biological functions. A WISP1 variant lacking exon 3 (WISP1v) loses its VWC domain, which is thought to participate in protein complex formation. Ectopic expression demonstrated that the WISP1v is a secreted oncoprotein, which drives cellular transformation and rapid cumulative growth. WISP1v overexpression enhanced the invasive phenotype in gastric carcinoma cells, while wild-type WISP1 exhibited no such potential. These findings suggested that the CCN protein WISP1v was involved in the aggressive progression of scirrhous gastric carcinoma (36). In CCA, the aberrant isoform WISP1v was demonstrated to be overexpressed in patient CCA tissues (37). Furthermore, upregulation of WISP1v was associated with shorter overall survival time among patients following surgical treatment (38). In addition, WISP1v was demonstrated to promote cell invasion *in vitro* and this process was demonstrated to be mediated by p38 MAPK (37).

Nek2A and Nek2B. Nek2, or NIMA-related kinase 2, is a serine/threonine kinase that regulates cell division through centrosome separation (39). The spliced isoform of Nek consists of three forms, Nek2A, Nek2B and Nek2A-T (40). Isoforms of NEK are demonstrated to be functionally involved with cancer formation. In patients, overexpression of Nek2a was associated with Ki-67 expression, a cell proliferation marker (41). In addition, NEK2A cytoplasmic expression was positively associated with cancer grade and tumor size in breast invasive ductal carcinoma and metastatic potential (42). Cancer cells overexpressing the NEK2A isoform demonstrated a significant increase in colony formation compared with control cells and small interfering (si)RNA-based depletion of NEK2a resulted in the halting of cancer cell proliferation (43). Nek2A/Nek2A-T were demonstrated to be highly upregulated in CCA cell lines, with the predominant isoform being Nek2A/Nek2A-T and Nek2B being the lesser expressed isoform (44). Furthermore, the expression of Nek2B was demonstrated to positively correlate with proliferation potential in breast cancer cells (45).

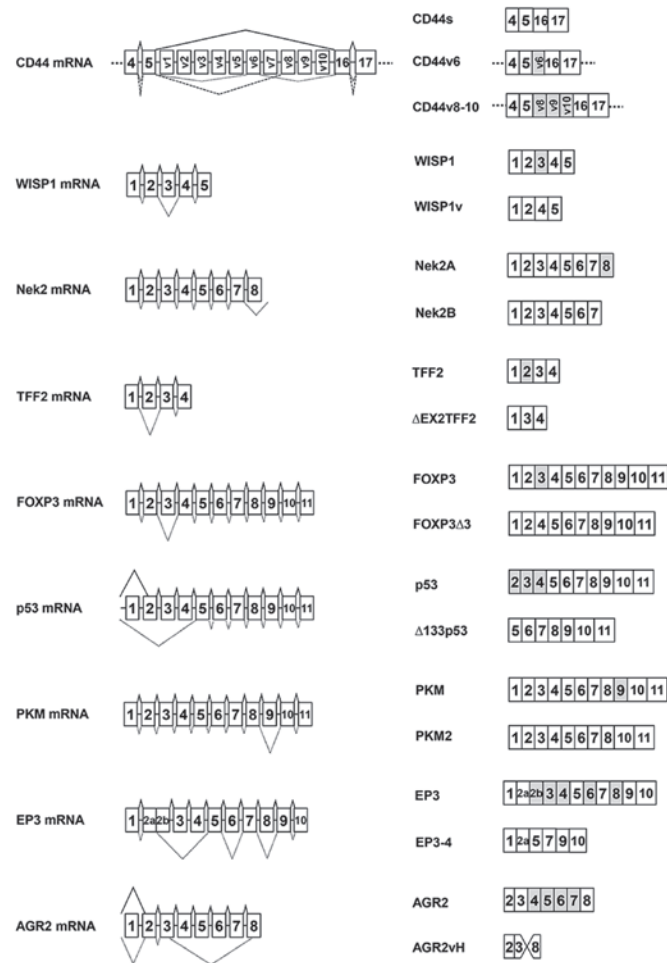


Figure 1. Schematic representation of the alternative splicing events implicated in cholangiocarcinoma development and progression. Exons are represented by boxes and introns by lines. Continuous lines represent the exon inclusion for wild-type mRNA, whereas dotted lines represent the exon inclusion for spliced transcripts. Skipped or included exons from alternative splicing, that differ from wild-type mRNA, are presented in gray.

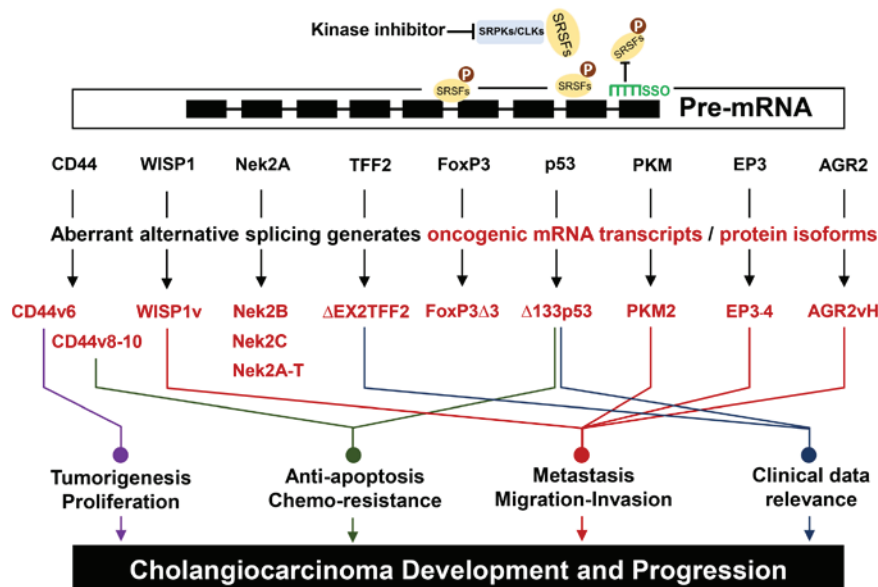


Figure 2. Spliced mRNA transcripts and their functions in cholangiocarcinoma.

ΔEX2TFF2. Trefoil factor 2 (TFF2) is a secreted protein that serves important roles in gastrointestinal restitution (46), chronic

kidney disease and pulmonary inflammation, through the induction of cell migration and proliferation. Overexpression of TFF2

Table I. Spliced mRNA transcripts and their functions in cholangiocarcinoma.

Author, year	Gene	Spliced transcript/ isoform	Splicing variants	Function	(Refs.)
Yun <i>et al</i> , 2002	CD44	CD44v6	Retained exon v6	Proliferation	(32)
Thanee <i>et al</i> , 2016	CD44v8-10	Retained exon v8-10	Anti-apoptosis		(33)
Tanaka <i>et al</i> , 2003	Wnt-inducible secreted Protein	WISP1v	Skipping exon 3	Neural and lymphatic invasion	(37)
Kokuryo <i>et al</i> , 2007	Serine/threonine-protein kinase Nek2	Nek2B	Skipping exon 8	Function unknown	(44)
Kamlua <i>et al</i> , 2012	Trefoil factor 2	Δ EX2TFF2	Skipping exon 2	Independent prognostic marker	(48)
Harada <i>et al</i> , 2012	Forkhead box protein 3	Foxp3 Δ 3	Skipping exon 3	Function unknown	(50)
Nutthasirikul <i>et al</i> , 2013	Tumor protein 53	Δ 133p53	Exon 1-4 skipping	Independent prognostic marker	(60)
Nutthasirikul <i>et al</i> , 2015				5-Fluorouracil resistance	(61)
Yu <i>et al</i> , 2015	Pyruvate kinase	PKM2	Mutually exclusive exons; exon 9 skipping and exon 10 retention	Neural invasion	(67)
Du <i>et al</i> , 2015	E prostanoid receptor 3	EP3-4	Exon 2b, 3, 4, 6 and 8 skipping	Proliferation migration and invasion	(71)
Yosudjai <i>et al</i> , 2018	Anterior Gradient-2	AGR2vH	Alternative 3' and 5' splice site and exon 4-7 skipping	Migration, invasion and adhesion	(74)

is commonly identified in several types of cancer, implicating it in carcinogenesis. TFF2 was reported to exert its pro-proliferative activity through the EGFR-MAPK pathway in CCA (47). Previously, Δ EX2TFF2, an exon 2- skipping isoform of TFF2 with a stop codon (TAG) at exon 1, was uncovered as a spliced isoform of TFF2 (48). Although, the roles of this transcript have not been clarified, the present study demonstrated that a high expression ratio of Δ EX2TFF2/wtTFF2 in patients was significantly associated with a longer survival time (48). Therefore, the spliced isoform may act as a dominant-negative form of TFF2 that counteracts the cancer promoting wtTFF2 activity in CCA.

Forkhead box protein 3 (FOXP3 Δ 3). FOXP3 is a transcription factor in the forkhead protein family that is involved in CD25⁺ regulatory T cell (Treg) development. Not only does FOXP3 control Treg development, it is also expressed in colorectal

cancer cells, which is associated with poor prognosis in patients (49). Exon 3 skipping of FOXP3, resulting in an amino acid frameshift, has been reported in CCA (50). In addition, a FOXP3 splice isoform was also observed in melanoma cells, suggesting it has a role in suppressing immune activity (51).

Δ 133p53. Tumor protein 53 (TP53 or p53) is one of the most important tumor suppressors, indicated by its high mutation rate across all types of cancer. p53 responds to various stress signals and orchestrates processes including cell cycle arrest, DNA repair, cellular senescence and apoptosis in response to specific stress signals (52). AS generates 12 p53 isoforms, including Tap53, Δ 40p53, Δ 133p53 and Δ 160p53 among others (53,54). The differential regulation of p53 isoforms promotes the aggressiveness of several types of cancer. A study demonstrated that Δ 133p53b enhanced breast cancer

stemness (55) and protected colorectal cells from camptothecin-induced apoptosis (56).

p53 has been identified as a gene that frequently mutates in a large number of CCA cases (57-59), suggesting that a perturbed p53 pathway facilitates CCA carcinogenesis. A study demonstrated that a high $\Delta 133p53/p53$ mRNA expression ratio correlates with a poor overall survival (60). Notably, $\Delta 133p53$ is also associated with resistance to certain cancer drugs; an association between $\Delta 133p53$ and 5-FU-resistance in CCA cells was demonstrated, and $\Delta 133p53$ was upregulated in 5-FU-resistant tumor tissues and CCA cell lines in a dose-dependent manner (61). Given that 5-FU is a cytotoxic drug that interferes with DNA synthesis, the $\Delta 133p53$ isoform may act as a dominant-negative p53 that interferes with the activity of wtp53 in the ternary complex (62). Accordingly, suppression of $\Delta 133p53$ promoted apoptosis, which correlated with an upregulation of pro-apoptotic Bax and a downregulation of anti-apoptotic Bcl-2 (61).

Pyruvate kinase (PKM2). PKM is a rate-limiting enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate during glycolysis. PKM can be generated in 4 isoforms, which are expressed differently in various tissues. One of the isoforms is PKM2, which lacks exon 9 and is a major isoform highly expressed in a number of types of cancer (63). Previously data demonstrate that overexpression of PKM2 is linked to tumor growth, metastasis capability and a poor prognosis in hepatocellular carcinoma, pancreatic ductal adenocarcinoma and gallbladder cancer (64-66). In hilar cholangiocarcinoma, immunohistochemical staining specific to the PKM2 isoform demonstrated a great number of positive-staining cells in the tumor tissue. Patients with high-PKM2-expressing tumors exhibited a higher rate of tumor recurrence and a shorter overall survival time, when compared with patients with low PKM2 expression. However, there is still no conclusive evidence that indicates PKM2 is a cancer driver for CCA. In addition, PKM2 elevation was associated with CCA development and neural invasion (67).

EP3-4. E prostanoid receptor 3 (EP3), or prostaglandin E2 receptor 3 (PTGER3), is a member of a G protein-coupled receptor family, that specifically binds to prostaglandin E2 (PGE2) to activate various responses. EP3 receptor can generate up to 11 spliced isoforms. Previous data demonstrate that EP3-5 and EP3-6 isoforms were associated with cell proliferation in the myometrium in humans (68). Furthermore, overexpression of the EP3-4 receptor promoted cell growth through upregulating FUSE-binding protein 1 in liver cancer (69). In CCA, the truncated EP3-4 isoform, which includes exon 1, 2a, 5 and 10, was detected (70). This EP3-4 isoform is activated through the Src/EGFR/PI3K/AKT/glycogen synthase kinase-3 β pathway and promotes cell proliferation, migration, and invasion. This results in enhanced expression of the downstream proteins c-Myc and snail. Therefore, it is believed to serve a regulatory role in CCA cell growth and metastasis (71).

Anterior Gradient-2 (AGR2)vH. The expression profiling of metastasis-associated genes in CCA demonstrated that AGR2 is one of the most-upregulated genes, specific to the metastatic CCA cell line, when compared with the parental cell line (72).

The AGR2 gene encodes for a disulfide isomerase enzyme, which is commonly expressed in mucus-secreting tissues. The mRNA splicing of AGR2 was first characterized in prostate cancer (PCa). Spliced isoforms include AGR2vC, AGR2vE, AGR2vF, AGR2vG and AGR2vH. Among the 5 spliced isoforms and the wild-type, AGR2vG and AGR2vH were demonstrated to be significantly upregulated in the exosome from patient's urine sample analysis. These two exhibited high diagnostic value, with higher sensitivity and specificity when compared with the prostate-specific antigen used as a standard clinical biomarker for PCa diagnosis (73). In CCA cell lines, AGR2 RNA isoforms, namely AGR2vE, AGR2vF and AGR2vH, were recently reported that are specific to metastatic CCA cells (74). It was demonstrated that the AGR2vH isoform enables various metastatic-associated phenotypes in CCA cells. Suppression of AGR2vH by the AGR2vH-specific siRNA significantly reduced CCA cell migration and invasion. Concordantly, AGR2vH overexpression promoted cell proliferation, migration, invasion and adhesion potential. In addition, it was demonstrated that the expression of AGR2vH influences metastasis-associated phenotypes through the upregulation of vimentin. Therefore, the results indicated that the metastasis-specific isoform AGR2vH serves an important role in cancer severity (74).

3. Targeting aberrant splicing as a novel concept for cancer treatment

The prominent role of the aberrant AS in carcinogenesis has been demonstrated, indicating that AS may be a good target for cancer therapy. Aberrant AS can be manipulated in several steps: For example, Pre-Trans-Splicing Molecule (PTM) is the artificial sequence that can reprogram mRNA through replacement of the 3'exon, 5'exon and internal exon (75,76). The results demonstrated that the trans-splicing molecule reduced the number of mutant p53 transcripts in the transfected cells, which resulted in cell cycle arrest, apoptosis and tumor xenograft suppression with colorectal cancer and hepatocellular carcinoma cells (77,78). However, the use of PTM for targeting oncogenic AS events is not yet well studied and the PTM modification has limitations for cancer treatment. Therefore, this review discussed the methodologies that may apply to cancer therapy, including small molecule splicing modulators and SSOs, each of which are currently under study in clinical trials.

Small molecules splicing modulators. Splicing factors are key molecules that influence AS regulation and are associated with cancer aggressiveness and pathological phenotypes (79). A previous report demonstrated that an overexpression of serine/arginine-rich splicing factor 1 (SRSF1) can facilitate abnormal splicing of tumor suppressors and proto-oncogenes (80). The results demonstrated that SRSF1 promotes 12A inclusion of an isoform of BIN1, which interferes with the tumor-suppressing activity of this protein. In the same study, the researchers demonstrated an increase in S6K1 isoform 2 expression resulting from SRSF1 overexpression that was associated with colony formation activity (80). An Ov-infected hamster model was used to identify the differentially expressed genes to study

the molecular mechanism of CCA carcinogenesis. The results demonstrated that SRSF9 is one of the genes that are overexpressed in Ov-infected hamsters and may be associated with CCA initiation (81).

Aberrant spliceosomal proteins are important factors associated with carcinogenesis. The data revealed that mutations in splicing factor 3B subunit 1A (SF3B1), which encodes the core component of U2 snRNP, is linked to erroneous 3' splice site selection (82-84). The results demonstrated that the SF3B1 K700E mutation led to differential splicing in uveal melanoma and breast cancer (85,86). In addition, luminal B and progesterone receptor-negative breast cancer patients with additional SF3B1 mutations have significantly shorter survival times (87).

It is possible to modulate aberrant AS based on small molecule inhibitors of splicing factors or mutated spliceosomal proteins: For example, it has been demonstrated that a natural product 'Borrelidin' can bind to a splicing protein, FBP21, leading to a decrease of the vascular endothelial growth factor (VEGF) pro-angiogenic isoform and an increase of the VEGF anti-angiogenic isoform, in RPE cells (88). Previous studies demonstrated that a natural product, FR901464 and its methylated derivative, spliceostatin A, as well as E7107, specifically inhibit spliceosome assembly through SF3B1 and lead to halted splicing reactions (89-91). The results demonstrated that treatment of these small molecules inhibits cell cycle progression and inhibits the tumor angiogenesis through decreasing the levels of VEGF transcripts (92,93).

Not only does the altered expression of splicing regulators affect AS, but the alteration of the phosphorylation status of the splicing factor/modulator was also implicated in cancer progression. In head and neck squamous cell carcinoma, hyperphosphorylation of SRPK2, a serine/arginine-rich protein-specific kinase that phosphorylates SRSF1/2, was detected in cancer cells; the phosphorylation promotes cell proliferation, migration and invasion (94). Alteration to the kinase alters the AS pattern. A previous study demonstrated that CLKs and SRSF protein kinases (SRPKs) are targets for kinase inhibitors to modulate AS; treatment with Cpd-1, Cpd-2, and Cpd-3 significantly reduced the levels of phosphorylated SR proteins, therefore affecting the splicing pattern of multiple genes and inducing cell apoptosis (95). Furthermore, the other kinase inhibitors, including ceramide, affect splice site selection of Bcl-x and increases pro-apoptotic isoforms through the dephosphorylation of the SR protein (96).

SSOs technology. SSOs are single-stranded nucleic acids, usually 15-25 bases, that are complementary to the mRNA target transcripts or the recognition sequence of the splice sites, that leads to modulated splicing. A number of studies demonstrated that SSO can inhibit aberrant RNA translation: I.e., MDM4 is the protein that contributes to embryonic development and is undetectable in adult tissues. An MDM4 isoform with exon 6 is frequently upregulated in cancer cells, impairing p53 tumor-suppressor function. The SSO-mediated skipping of exon 6 results in decreased MDM4 levels and reduced melanoma growth (97). Similarly, SSO targeting exon 26 of HER4 mRNA, named as SSOe26, demonstrated its capacity on HER4 isoform switching from CYT1 to CYP2. This treatment resulted in the depletion of the proliferation of breast cancer

cells and tumor growth in mice xenografts (98). Furthermore, SSO targeted B-cell lymphoma (Bcl)-x pre-mRNA, which increased the Bcl-xS isoform, gaining pro-apoptotic activity, which was verified in the models of murine melanoma and in human glioma cell lines (99,100).

Drug development based on targeting aberrant AS, namely small molecule splicing modulators, is an interesting approach for cancer treatment. Splicing regulators are upstream molecules that control the splicing events of multiple genes. Insight into novel target genes of the splicing regulators, can be used to manipulate the effective inhibitor(s) of these upstream molecules to suppress various downstream oncogenic spliced isoforms. However, the off-target effect, toxicity (101,102) and the effects of small splicing factors interfering with the normal splicing patterns of global genes, should be considered. On the other hand, the specificity of SSO technology overcomes more than small splicing modulators by modulating AS through inhibiting only its oncogenic target which leads to effective treatment. The major problems of oligonucleotides include toxicity, instability against nucleases and delivery limitations.

4. Conclusion

The present review summarized the experimental evidence for and clinical relevance of the verification of significant effects of aberrant mRNA splicing of well-characterized genes with respect to CCA initiation and aggressiveness. The nine genes discussed underwent AS and revealed an intercorrelation with cholangiocarcinogenesis and progression. This information will serve as an opportunity to develop novel strategies for CCA detection and intervention. Interestingly, certain of the cancer-specific variants may serve as potential targets for CCA prognosis including $\Delta 2TFF2$ and $\Delta 133p53$, which demonstrate their clinical impact on patient survival. These oncogenic isoforms may be used as targets for cancer treatment, using specific antibodies, or the construction of SSOs which can modulate aberrant splicing. The regulatory machinery, including splicing factors and regulators, represents alternative targets of precision strategies, regarding the depletion of oncogenic isoforms. Finally, this summarization provides new ideas for the improvement of CCA diagnosis and treatment. Further studies should aim to investigate the unclear linkages between AS and CCA to unlock the molecular mechanisms governing AS regulation in CCA development and progression.

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Authors' contributions

JY and WK designed, performed and wrote the literature review. SJ and SW revised the manuscript for intellectual content.

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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