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Sonic Hedgehog signaling pathway as a potential target to inhibit the progression of hepatocellular carcinoma (Review)

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Abstract. Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-associated mortality worldwide. Hepatocarcinogenesis involves numerous interlinked factors and processes, including the Sonic hedgehog (Shh) signaling pathway, which participates in the carcinogenesis, progression, invasiveness, recurrence and cancer stem cell maintenance of HCC. The Shh signaling pathway is activated by ligands that bind to their receptor protein, Protein patched homolog (Ptch). The process of Shh ligand binding to Ptch weakens the inhibition of smoothened homolog (SMO) and activates signal transduction via glioma-associated oncogene homolog (Gli) transcription factors. The overexpression of Shh pathway molecules, including Shh, Ptch-1, Gli and SMO has been indicated in patients with HCC. It has also been suggested that the Shh signaling pathway exhibits cross-talk between numerous other signaling pathways. The inactivation of the Shh signaling pathway reduces HCC growth, increases radio-sensitivity and increases the beneficial effect of chemotherapy in HCC treatment. Therefore, inhibition of the Shh pathway may be an effective target therapy that can be used in the treatment of HCC.

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Abbreviations: HCC, hepatocellular carcinoma; Shh, Sonic Hedgehog; Ptch, Protein Patched homolog; SMO, Smoothened; Gli-1, glioma-associated oncogene homolog 1; HBV/HCV, hepatitis B or C viral; Hh, Hedgehog; Sufu, suppressor of fused homolog; EMT, epithelial-mesenchymal transition; MMP-9, metalloproteinase-9; SASH1, SAM- and SH3-domain containing 1; TGF, transforming growth factor; DEN, diethylnitrosamine; sFRP1, secreted frizzled related protein 1; MeCP2, methyl-CpG binding protein 2

Key words: sonic hedgehog signaling pathway, hepatocellular carcinoma, recurrence, cancer stem cells

Contents

1. Introduction

Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer-associated mortality worldwide, and involves numerous interlinked factors such as inflammation, hypoxia, immunity and processes from liver injury, liver cirrhosis to hepatocarcinogenesis (1). The well-established etiological factors include chronic hepatitis B or C viral (HBV/HCV) infection, excessive alcohol intake, non-alcoholic steatohepatitis and exposure to aflatoxin B (2-5). In Asia, the prevalence of HBV-infected HCC is 50-80% from 2016 (6,7). HBV/HCV infection can lead to apoptosis and immune cell infiltration, and can cause the induction of damaging inflammatory responses and repeated chronic tissue injury and repair, which results in the progression of HCC (8).

Currently, the most effective treatments for HCC are hepatic resection or liver transplantation (9). However, less than 20% of patients with HCC can be treated surgically. Furthermore, the 5-year recurrence rates of HCC after surgical resection were 57-75% due to the limited efficacy of chemotherapy, radiation and target therapy (10). Consequently, there is an essential requirement for the development of improved therapeutic strategies that can be used to treat HCC. The investigation of the signaling pathways driving hepatocarcinogenesis could be helpful to identify novel targets for HCC treatment. Hedgehog (Hh) signaling pathway contributes to the progression of a variety of human cancer types, including HCC, breast cancer and basal cell carcinoma (11-14). The Hh pathway serves a crucial role in carcinogenesis, invasiveness, recurrence and cancer stem cell maintenance in HCC (12). In the current review, recent studies of investigating the Shh signaling pathway and its association with HCC, were assessed.

2. Hh signaling pathway

Hh molecules are important soluble factors that regulate cell proliferation and differentiation during embryonic development, adult tissue homeostasis and carcinogenesis (15,16). Molecules that are associated with Hh signaling were first identified in *Drosophila*, and later found to be highly conserved in higher organisms, including mammals (17). In human, three types of Hh ligands have been associated with this pathway, Sonic hedgehog (Shh), Indian hedgehog and Desert hedgehog. In the liver, Hh ligands generated by injured hepatocytes, activated hepatic stellate cells, progenitor cells, Kupffer cells, natural killer T cells and endothelial cells (18). Each Hh ligand exhibits a different spatial and temporal expression patterns (19). The roles of these ligands in cellular and developmental function has been previously reviewed (20). In the current review, the role of the Shh signaling pathway in HCC was evaluated. The Shh signaling pathway is activated when Shh ligands bind to their receptors, protein patched homolog (Ptch) 1 or 2 (21,22). In the absence of Hh ligands, Ptch continuously inhibits the activity of G-protein-coupled receptor like receptor, SMO protein, in the Off stage (Fig. 1, left) (23). Suppressor of fused homolog (Sufu) is an important negative regulator of the Hh signaling pathway (24). Sufu sequesters glioma-associated oncogene homolog (Gli) transcription factors in the cytosol prior to pathway activation (25). The binding of Hh ligands to Ptch weakens the inhibition of SMO and promotes its translocation to the plasma membrane for complete activation. The activation of SMO results in the dissociation of the Gli-Sufu complex, and leads to the translocation of Gli protein to the nucleus (Fig. 1, right). There are three Gli family transcription factors: glioma-associated oncogene homolog 1 (*GLI-1), GLI-2* and *GLI-3* (26). Gli-1 and Gli-2 are transactivators and Gli-3 is a repressor. In the nucleus, the Gli proteins bind to the Gli-binding consensus sequence and upregulate the expression of target genes, including *SNAIL*, *c-MYC*, *BCL-2* and *Prominin-1* (CD133) (27).

3. Overexpression of the Shh signaling pathway in HCC is associated with a poor prognosis

The activation or overexpression of Hh molecules, including PTCH-1, GLI and SMO have been frequently indicated in HCC tissues (28-30). Che *et al* (28) analyzed 46 HCC tissues using reverse transcription-quantitative PCR (RT-qPCR) and identified numerous Hh signaling molecules that were expressed in ≥50% of tumors. Specifically, *GLI-1* expression was associated with disease-free and overall survival in patients with HCC. Therefore, *GLI-1* expression may be a prognostic predictor of HCC. Another study used RT-qPCR to analyze 50 patients with HCC after surgical resection, and it was demonstrated that high *PTCH-1* and *GLI-1* expression increased the risk of post-section recurrence and was associated with poor overall survival (29). The results of this study indicated that higher *PTCH-1* expression was associated with the early recurrence of HCC and may serve as a poor prognostic marker for the disease. Another study measured *GLI-2* levels, using immunohistochemistry in 68 patients with HCC. The results revealed that the patients with increased *GLI-2* expression exhibited earlier HCC recurrence, and presented with both shorter disease-free and overall survival times (31). In summary, these studies demonstrated that the activation of the Shh signaling pathway in patients with HCC was inversely associated with disease prognosis.

4. Various factors activate the Shh pathway in HCC

A number of studies have demonstrated that an association exists between the activation of the Shh signaling pathway and poor prognosis in patients with HCC. Therefore, it is important to understand how the pathway is activated. Adult healthy hepatocytes barely express Hh ligands, however, live epithelial cells begin to generate Hh ligands during injury or severe stress. The overexpression of Shh ligands and the concomitant expression in HCC tissues can activate the Shh signaling pathway (28,32,33). In addition, viral infection and chronic inflammation play a critical role in the activation of the Shh signaling pathway. HBV DNA integrates into hepatocyte chromosomes. The expression of the *HBV* gene product HBx protein can increase *SHH*, *PTCH-1* and *GLI-2* expression, and is able to stabilize GLI-1 and GLI-2 proteins (34,35). Hh signaling pathway activity has been indicated to be necessary for HBx transformation, as demonstrated by a study that showed that the administration of a SMO inhibitor reduced HCC growth in HBx transgenic mice (35). HBV/HCV infection has also been indicated to increase Hh ligands in hepatocytes and expand the Hh-responsive cells, which promote liver fibrosis and hepatocarcinogenesis (36). Chronic infection with HBV or HCV leads to continuous hepatocyte apoptosis, leukocyte infiltration and stimulates the Hh signaling pathway (3,35,37). A novel mouse model has revealed that chronic or acute liver injury can induce the activation of the Hh pathway. In this aforementioned study, primary hepatocytes upregulated SMO expression during Fas-induced liver injury and this also increased Fas-induced apoptosis (38). Activation of the Hh pathway initiates downstream gene expression of cancer stem cell marker CD133 and cytokine IL-6, which serves an important role in the liver acute phase response and in HCC development (39-41). PTCH-1 has been revealed to regulate cell cycle progression and induce tumorigenicity (42). Inhibition of the Hh pathway can reduce PTCH-1-dependent tumor progression (43). In addition to viral infection, mutation of the Hh signaling pathway molecules can also activate the Hh pathway (14). In a number of patients with HCC, SMO mutation at the C-terminal lysine (K575M) was

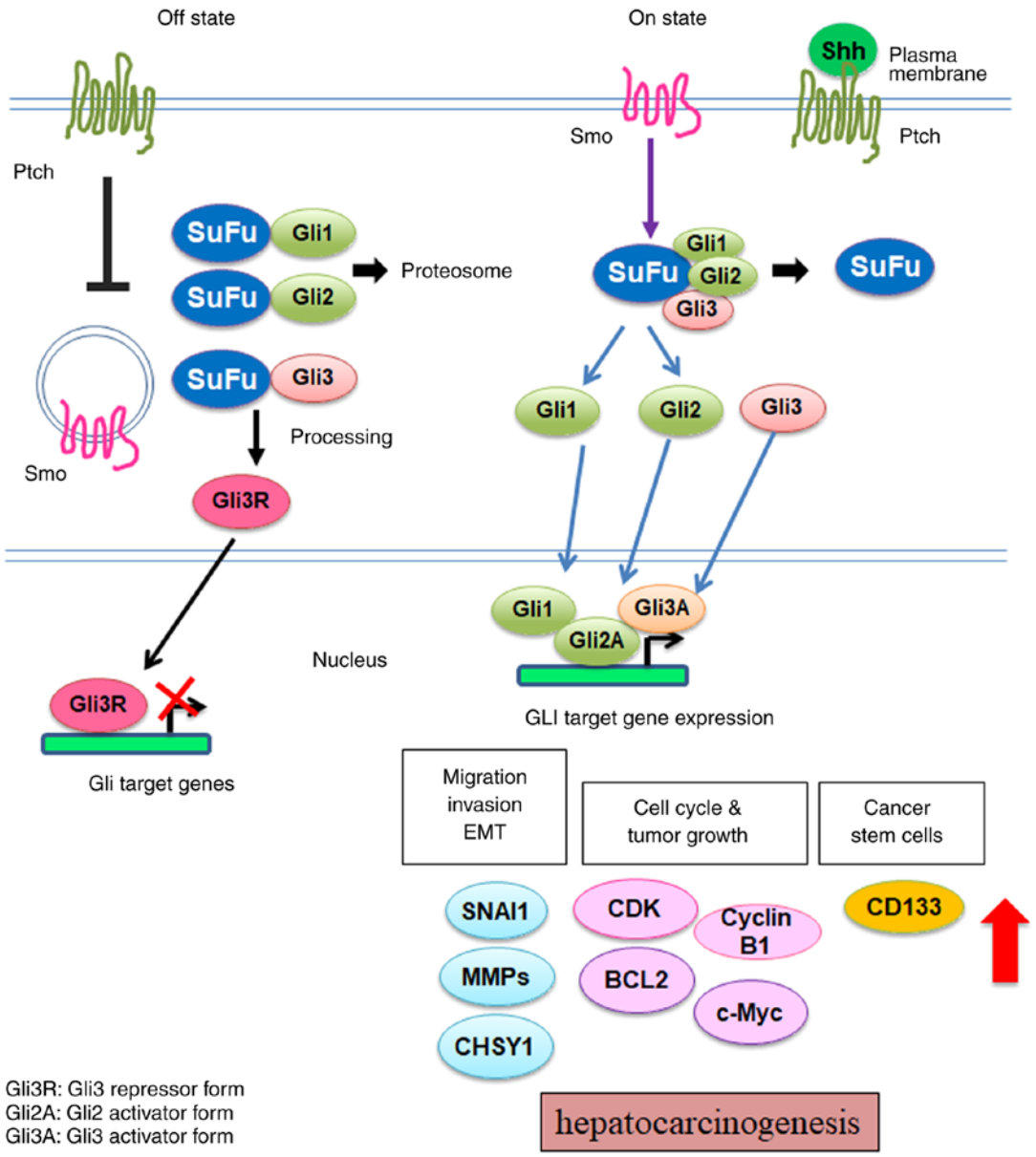

Figure 1. Shh signaling pathway in hepatocellular carcinoma. Left (Off state): Ptch inhibits SMO activation. The signaling from SMO to Gli is blocked. SuFu binds to Gli for protein degradation via the proteasome. Therefore, the expression of Hh signaling pathway target gene turns off. Right (On state): Shh ligand bind to their receptor, PTCH. The binding of Hh ligands to PTCH weakens the inhibition of SMO and activates signal transduction via GLI transcription factor, activating downstream gene expression in cell migration/invasion/transition (SNAIL, MMPs, CHSY1), cell cycle/tumor growth (cyclin B1, CDK, Bcl2) and cancer stem cell marker (CD133). Shh, Sonic hedgehog; Ptch, protein patched homolog; SMO, smoothened homolog; Gli, glioma associated oncogene homolog; EMT, epithelial-mesenchymal transition.

revealed to alter the binding between PTCH and SMO, alleviating SMO from PTCH inhibition and subsequently activating downstream signaling (44).

5. Activation of the Shh pathway enhances hepatocarcinogenesis and HCC progression

Accumulating evidence has demonstrated that an activated Shh pathway is associated with hepatocarcinogenesis (11,12,32). The Shh pathway is activated in tumors and differentiates cancerous cells from the non-cancerous cells (45). In a transgenic mouse model, Shh expression in the liver was indicated to, not only induce liver fibrosis, but also enhance hepatocarcinogenesis (46). Activated Shh signaling increases cyclin B1 and cyclin-dependent kinase 1 (CDK1) protein expression, which facilitates the G_2/M transition to promote cell proliferation and enhance hepatocarcinogenesis (47). In addition, the overexpression of *SMO* and an increased ratio of *SMO* to *PTCH* mRNA expression is associated with tumor sizes in human HCC, and SMO-mediated c-Myc overexpression serves a crucial role in HCC development (32). SMO is an important regulator of adult liver repair due to its role in the promotion of epithelial-mesenchymal transition (EMT), and serves a key role in the early stages of HCC development (48). Lastly, GLI transcription factors are also involved in HCC formation. GLI-2 serves a dominant role over GLI-1 or GLI-3 in promoting HCC cell proliferation and survival (49). This is consistent with the observation that enhanced GLI-2 expression

is associated with early recurrence and shorter survival in patients with HCC (31). Therefore, it can be suggested that the Shh signaling pathway is essential for the development and progression of HCC. Shh pathway may promote HCC growth via upregulating genes that promote cell cycle (*CYCLINS, CDK* and *c-MYC*) and cell survival (*BCL2*).

6. Activation of Shh signaling pathway contributes to invasiveness, metastasis and behavior of cancer stem cells

In addition to inducing HCC growth, evidence has also demonstrated that an activated Shh signaling pathway is associated with HCC capsular/vascular invasion (28,31). The Shh signaling pathway mediates HCC invasion and metastasis by upregulating matrix metalloproteinase-9 (MMP-9) (50). Bromodomain 4, which is a transcriptional and epigenetic regulator, enhances HCC cell migration and invasion through Shh signaling pathway-mediated MMP-2 and MMP-9 activation (51). The upregulation of GLI-1 expression in HCC tissues is associated with clinicopathological characteristics (52). Therefore, GLI-1 may participate in HCC progression and metastasis via the induction of EMT. Twist is a key transcriptional factor for EMT, which promotes the invasion and metastasis of tumor cells (53,54). Increased Gli-1 and Twist expression has been observed in HCC tissues, indicating the possible involvement of the Shh pathway in EMT (55). The Shh pathway promotes HCC migration and invasion through activation of FAK and AKT, and the subsequent upregulation of MMP-2 and MMP-9 expression (56). Based on the available evidence, it can be concluded that the Shh pathway can regulate invasion, migration and EMT, at least in part, via regulating MMP expression.

SAM- and SH3-domain containing 1 (*SASH1*) is a tumor suppressor gene that belongs to the SLY family adaptor proteins (57). SASH1 may reduce cancer cell proliferation, migration and invasion (58). SASH1 has been demonstrated to downregulate the Shh-Gli-1 and PI3K-AKT pathways to inhibit HCC invasion and metastasis, underlying the importance of the Shh pathway in HCC progression (59). CHSY1 encodes for an enzyme that catalyzes the polymerization of chondroitin sulfate (60,61). The overexpression of CHSY1 enhances HCC migration, invasion and EMT by promoting Shh binding and signaling (62). One study revealed that treatment with Vismodegib, a SMO inhibitor, decreased CHSY1-induced HCC cell migration, invasion, and lung metastasis (39). In summary, the Shh pathway regulates cell growth, and can contribute to the invasion and migration of HCC cells.

The Shh signaling pathway can influence the behaviors of cancer stem cells (63). Shh signaling pathway activation occurs in cancer stem cells ($CD133^+$) of mouse hepatoma cell line Hepa1-6. $CD133^+$ HCC cells with upregulated SMO mRNA exhibit significantly higher colony proliferation and clonogenicity compared with that in $CD133^-$ HCC cells (64). However, whether the Shh pathway controls these behaviors in the cancer stem cells of human HCC, requires further investigation.

7. Shh signaling pathway cross-talks with other signaling pathways

Transforming growth factor (TGF)-β serves a key role in the induction of EMT and its expression has been revealed to be elevated in 40% of human HCC tissues (65,66). The activation of the Shh signaling pathway is frequently detected in HCC; therefore, the Shh signaling pathway may interact with TGF-β signaling to enhance EMT in HCC (28). Using computational algorithms and confirmation of its presence in HCC cell lines, Steinway *et al* (67) demonstrated that TGF-β induced the activation of Wnt and Shh signaling to regulate EMT in HCC. Furthermore, GLI-2 has been identified as a direct target of TGF-β/SMAD signaling pathway in a variety of cell types, including fibroblasts, breast cancer cells and pancreatic carcinoma (68). *GLI-2* expression is associated with the expression of active forms of SMADs in HCC tissues (67). Therefore, it can be suggested that TGF-β may activate the Shh signaling pathway via upregulation of GLI-2. TGF-β is significantly elevated with the active form of SMO in mouse keratinocytes, suggesting that the Hh pathway can increase TGF-β expression (69). Therefore, the expression of Shh and TGF-β signaling together, may amplify each other and participate in the invasion and metastasis of HCC.

In other varieties of primary and tumor cells, active Hh signaling has been demonstrated to induce EMT via WNT, EGF/FGF, Notch and TGF-β signaling cascades (70). The Wnt/β-catenin pathway is a well-known promoter of HCC development, and it is possible that this pathway cross-talks with the Shh signaling pathway to regulate HCC progression (71). A recent study demonstrated how the cross-talk between the Hh and Wnt pathway can contribute to HCC formation in a diethylnitrosamine (DEN) -administrated obese mouse model (72). In chronic fibrosis, upregulated Gli could target Myc to drive TGF-β2 expression for Wnt5a secretion (72). Receptors for Wnt5a are highly expressed in mouse HCC and a number of poorly differentiated human cell lines (72). Furthermore, elevated Wnt5a expression has also been detected in poorly differentiated human HCC cells, suggesting that both Hh and Wnt ligands are able to function in an autocrine-positive feedback manner to maintain tumors (72). In the DEN-induced HCC model, Wnt signaling and Hh pathway activity increased during HCC development (73). An increased Gli-1 and Gli-2 expression is associated with an increase in Wnt pathway inhibitor-secreted frizzled related protein 1 (sFRP1) in HCC (73). It has also been found the upregulation of sFRP1 and Gli is present in patients with intermediate and advanced HCC (73).

8. Targeting the Shh signaling pathway as a promising treatment strategy for HCC

Accumulated evidence has demonstrated that the Shh pathway is associated with the growth, invasiveness, recurrence and cancer stem cell maintenance of HCC (12,29,30). Therefore, inhibition of the Shh signaling pathway may be used as a potential HCC therapeutic strategy. The inhibition of Hh signaling may inhibit liver fibrosis and decrease cancer progenitors (74). Hh antagonist GDC-0449 and cyclopamine have been revealed to bind to SMO and inhibit the Shh signaling pathway (75,76). In a HBx transgenic mouse model, the inhibition of the Hh signaling pathway by GDC-0449 delayed hepatocarcinogenesis (35). The results of previous studies showed that the SMO inhibitors cyclopamine and GDC-0449 reduced HCC growth and immune infiltration *in vivo*, and tumor size and Gli-1

Table I. Functions/features of Shh signaling pathway molecules in HCC.

Molecule	Function/features	Model	Study	(Refs.)
Shh	HCC progression, prognostic predictor of HCC	Human	Che, 2012	28
	Induce liver fibrosis and enhance hepatocarcinogenesis	Mice	Chung, 2015	46
Ptch	Regulate cell cycle	Mice	Adolphe, 2006	42
	Postresection recurrence	Human	Jeng, 2013	29
Smo	HCC cell migration, tumor development	Mice	Arzumanyan, 2012	35
	Regulator of adult liver repair, early stage of HCC development	Mice	Michelotti, 2013	48
	Smo mutation (K575M) alter the binding between Ptch and Smo	Human	Ding, 2014	44
	Deletion of Smo enhances Fas-induced liver injury	Mice	Wang, 2018	38
Gli-1	HCC progression, prognostic predictor of HCC	Human	Che, 2012	28
	Postresection recurrence	Human	Jeng, 2013	29
	Tumor formation	Mice	Wang, 2013	80
	Associated with Twist expression for EMT	Human	Chun, 2016	55
Gli-2	HCC proliferation and survival	Human	Kim, 2007	49
	Poor survival, poor prognostic, recurrence	Human	Zhang, 2013	31

Shh, Sonic hedgehog; Ptch, protein patched homolog; SMO, smoothened homolog; Gli, glioma associated oncogene homolog; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma.

mRNA expression were significantly decreased (77,78). Thus, GDC-0449 treatment is effective in reducing HCC tumor sizes and the degree of cell infiltration (immune cell recruitment) in mouse models.

Gli may also be another therapeutic target. The inhibition of GLI-1 suppresses cell growth/cell cycle progression, induces apoptosis as well as autophagy via an Erk1/2 activity-dependent mechanism in human chondrosarcoma (79). Wang *et al* (80) revealed that the GLI inhibitor GANT61 inhibited tumor formation and decreased tumor sizes in a Huh7 xenograft model. The autophagy inhibitor, 3-MA, partially blocked this effect. Results of the aforementioned study suggested that inhibition of the Hh pathway may induce autophagy through the upregulation of Bcl2-interacting protein 3, which displaces Bcl2 from Beclin-1 to induce apoptosis. Therefore, autophagy status is a crucial factor to determine the therapeutic responses to Hh-targeted therapies. Other inhibitors that target the Hh signaling pathway, including XL-139, IPI-926 and LDE-225, were tested in clinical trials (81).

Melittin, a bee venom, inhibits HCC cell proliferation by downregulating Methyl-CpG binding protein 2 (MeCP2) via the Shh signaling pathway (82). Melittin treatment may increase Ptch expression due to its induction of the demethylation of Ptch1 promoter, which is associated with the downregulation of MeCP2 (82). Furthermore, the downregulation of Shh and Gli-1 has also been revealed in Melittin treatment (82). In addition, the downregulation of Gli-1 alone was not sufficient to inhibit HCC cell proliferation, and the downregulation of Gli-2 decreased both Gli-1 and other target gene expression. These data suggest that Gli-2 may regulate the expression of numerous downstream genes (49). The siRNA-mediated silencing of *GLI-2* significantly reduced HCC cell proliferation, therefore, GLI-2 may be a novel target that can be used in the regulation of HCC growth (49).

It has been suggested that activated Hh signaling may protect human HCC cells from radiotherapy, and that cyclopamine is a potential radiosensitizer (83,84). Shh ligand has been demonstrated to exhibit a protective effect on clonogenic cell survival upon irradiation treatment in HCC cells (84). The combination of irradiation and cyclopamine may be a more effective way to inhibit HCC cell proliferation than using either modality alone. The suppression of cell proliferation by cyclopamine may be attributed to an increase in apoptosis. Radiation upregulates Shh expression in a dose-dependent manner and increases Gli-1 expression in the nucleus (84). Irradiation with cyclopamine treatment could inhibit Gli-1 and increase the breakdown of DNA. In a previous study, when compared with radiotherapy alone, cyclopamine with radiotherapy reduced tumor sizes more effectively. Therefore, radiotherapy with a Shh inhibitor may increase the radio-sensitivity of HCC cells (84). Furthermore, 5-FU treatment may downregulate the expression of Shh signaling and inhibit motility in hedgehog-activated HCC cell lines (85). These results suggest that 5-FU-based chemotherapy with a Shh signaling pathway inhibitor could be a promising treatment option for HCC.

9. Conclusions and future direction

HCC is the most common liver malignancy worldwide. Despite advancements in diagnostic methods such as ultrasound, multi-detector computed tomography, magnetic resonance imaging and biomarkers and surgical techniques, the recurrence rates after surgical resection (57-75%) or liver transplantation (15-20%) remain high, and early recurrence decreases the survival rates of patients with HCC (9,10,86). Predicting early recurrence in patients after resection remains a challenge. For patients with a high chance of recurrence, postoperative adjuvant therapies are required. The Shh signaling pathway is highly activated in patients with HCC, affecting hepatocarcinogenesis, HCC progression, cancer stem cell maintenance, invasion and HCC recurrence (Table I). Inhibiting the Shh pathway could therefore be an effective target therapy for HCC treatment.

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Authors' contributions

All authors prepared literatures, revised and approved the final manuscript. KSJ, CML, YGT and CFC performed the literature search, wrote/edited the manuscript and prepared the figure and the table. CJJ, WJJ, ISS and SYL contributed to literature review and the conception of the study.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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