Review

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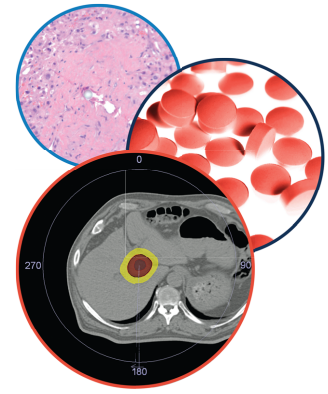

Hepatic Oncology

JAK/STAT signaling in hepatocellular carcinoma

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Liver cancer is the second most lethal cancer in the world with limited treatment options. Hepatocellular carcinoma (HCC), which accounts for more than 80% of all liver cancers, has had increasing global incidence over the past few years. There is an urgent need for novel and better therapeutic intervention for HCC patients. The JAK/STAT signaling pathway plays a multitude of important biological functions in both normal and malignant cells. In a subset of HCC, JAK/STAT signaling is aberrantly activated, leading to dysregulation of downstream target genes that controls survival, angiogenesis, stemness, immune surveillance, invasion and metastasis. In this review, we will focus on the role of JAK/STAT signaling in HCC and discuss the current clinical status of several JAK/STAT inhibitors.

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Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the second most common cause of cancer-related deaths worldwide [1]. The global incidence of HCC has been increasing, with an estimated range of 600,000–800,000 new cases occurring annually [2–4]. HCC is a highly heterogenous disease with multiple risk factors and etiologies, including chronic hepatitis B or hepatitis C virus (HBV/HCV) infections, excessive alcohol consumption, aflatoxin exposure and diabetes or obesity-related metabolic syndromes, that vary depending on the geographic distribution [5]. Generally, the dominant risk factors for HCC in high incidence rate countries such as those in Asia and Africa are HBV infections and aflatoxin B exposure, whereas HVC virus infections, alcohol consumption and metabolic syndromes are more important risk factors in low incidence regions, which include countries in Europe, North and South America and the Middle East [6,7]. Nevertheless, about 70–80% of HCC cases develop from a background of liver cirrhosis, with a median time to development of 10 years [8–10]. The underlying diseased/cirrhotic liver contributes to the poor prognosis/high mortality of many HCC patients, along with the difficulty of early diagnosis and a lack of effective late-stage treatment options.

Currently, the types of interventions available for treatment of HCC vary depending on the stage of the disease. Early-stage HCC patients are amenable for potentially curative treatments, such as surgical resection and liver transplantation, while patients with intermediate stage HCC are often given locoregional therapies, which include radiofrequency ablation, transarterial chemoembolization and radioembolization [11]. Unfortunately, many HCC cases often present at advanced and unresectable stages and systemic therapy is usually the only viable option for such patients [12,13]. Currently, the clinical standard of care systemic treatment for advanced HCC is the small molecule inhibitor sorafenib, a multi-kinase inhibitor targeting RAF, VEGFR 2, VEGFR 3, PDGFRβ, c-KIT, FLT-3 and RET [14,15]. Since its approval by the US FDA in 2007, sorafenib has been the sole systemic drug for HCC for more than a decade. However, in recent years, several other drugs have also been approved for advanced HCC, including lenvatinib (an alternative first-line treatment), regorafenib, cabozantinib and ramucirumab (second-line treatments for sorafenib-refractory patients) [16–19]. Lenvatinib, regorafenib and cabozantinib are multi-kinase inhibitors, like sorafenib, whereas ramucirumab is a monoclonal antibody against VEGFR2. Important targets of lenvatinib include VEGFR 1-3, FGFR 1-4, PDGFRα, RET and c-KIT; for regorafenib, VEGFR 1-3, PDGFRβ, FGFR 1, c-KIT, RET and B-RAF; and for cabozantinib, VEGFR 1-3, MET and AXL.

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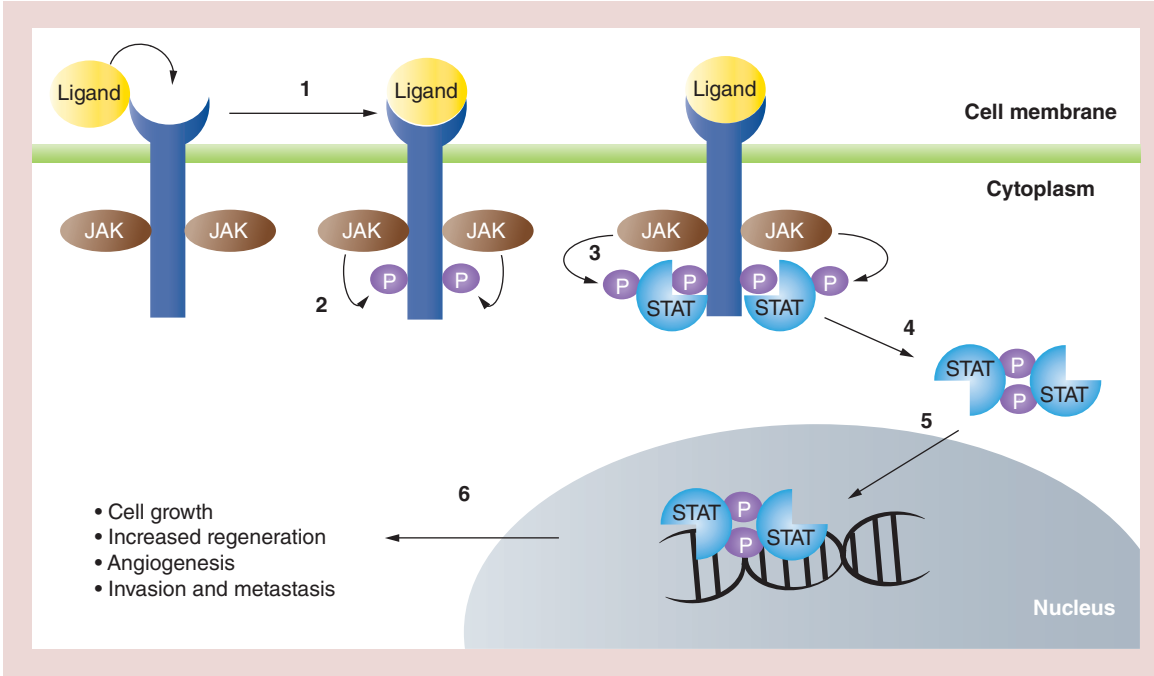

Figure 1. JAK/STAT signaling pathway overview. (1) Ligands such as cytokines and growth factors bind to transmembrane receptors, activating receptor-associated JAKs. **(2)** JAKs phosphorylate cytoplasmic tails of receptors, **(3)** recruiting STATs to the receptor and become phosphorylated by JAKs. **(4)** Activated STATs dimerize and **(5)** translocate into the nucleus where they bind to DNA and **(6)** activate transcription of target genes such as those involved in regulating cell growth.

Nevertheless, while these drugs have shown clinical benefits, the improvements in patient survival and outcome remain marginal [5]. The effectiveness of these treatments has also been hampered by the development of drug resistance and underlying liver dysfunction [12,20]. Even for curative treatments, disease recurrence represents a major drawback, with a 5-year incidence rate of over 70% [21]. These factors explain why HCC remains a highly difficult cancer to treat. Hence, there is an urgent need to develop better therapeutic strategies, especially for advanced HCC patients. In this regard, given the multitude of molecular signaling pathways that contribute to HCC development, targeted therapy based on identification and understanding of these molecular mechanisms provides a promising alternative/approach for treatment of HCC.

JAK/STAT signaling pathway

Aberrant activation of various intracellular signaling pathways involved in cell growth, differentiation, apoptosis and survival have been found to contribute to HCC development and progression [22,23]. These include known oncogenic signaling pathways such as Wnt/β-catenin pathway, PI3K/Akt/mTOR pathway, Ras/Raf/MAPK pathway and JAK/STAT pathway [23].

The JAK/STAT signaling pathway plays important roles in many cellular functions, including cell proliferation, stem cell maintenance and differentiation as well as modulation of the immune/inflammatory response [24]. JAK/STAT signaling has also been reported to regulate liver regeneration and gluconeogenesis [25]. The JAK/STAT pathway can be activated by various cytokines and growth factors, such as interleukins, interferons and EGF family members, which bind to their respective transmembrane receptors. The cytoplasmic tails of some of these receptors are associated with Janus kinases (JAKs) that become activated upon ligand-induced conformational change of the receptors. These activated JAKs then phosphorylate tyrosine residues on the cytoplasmic tail of the receptor, creating docking sites for a family of signal transducers and activators of transcription, known as STATs. Upon binding to the receptor, these STATs are phosphorylated by JAKs, become activated and form dimers, which then translocate to the cell nucleus. Subsequently, the STAT dimers recognize and bind to specific promoter sequences to activate transcription of their target genes, for example, *CCND1*, *BIRC5* and *Mcl-1* (Figure 1) [26].

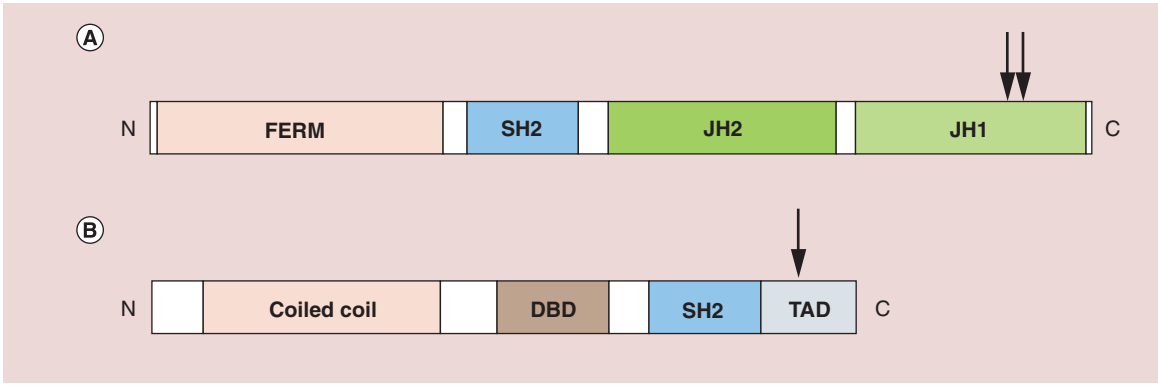

Figure 2. Schematic structures of JAK and STAT proteins. (A) JAK proteins contain a FERM domain that associates with receptors, a SH2 domain that binds phosphorylated tyrosine residues and two kinase domains JH1 and JH2. Arrowheads indicate phosphorylation sites (tyrosine residues) required for JAK activation. **(B)** STAT proteins contain a coiled coil domain for dimerization, a DBD, a SH2 domain and a TAD for transcriptional activation of target genes. Arrowheads indicate the conserved tyrosine residue that needs to be phosphorylated for STAT activation. N and C represents the amino- and carboxy-terminal ends respectively.
DBD: DNA-binding domain; TAD: Transactivation domain.

In humans, there are four members in the JAK family – JAK1, JAK2, JAK3 and TYK2. The JAK proteins contain two adjacent kinase domains that serve different functions (Figure 2A). JH1 domain performs the typical phosphorylation of STATs and receptors, while the JH2 domain regulates JH1 [24]. Additionally, JAKs also contain a FERM domain (4.1 protein, ezrin, radixin and moesin) that is responsible for interacting with receptors and a SH2 (Src homology 2) domain that binds to phosphorylated tyrosine residues [27,28].

The human STAT protein family comprises of seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. These proteins share several functional domains, including the SH2 domain, which recognizes phosphorylated tyrosine residues on the receptors, and activated STAT proteins, and a coiled-coil domain, which enables dimerization of activated STATs as well as interaction with other proteins (Figure 2B) [27]. In addition to binding to DNA, the DNA-binding domain is also involved in nuclear translocation of STAT dimers. STATs also contain a C-terminal transactivation domain necessary for activation of transcription.

Homeostatic regulation of JAK/STAT signaling is mediated by negative regulators that work at multiple levels of the pathway. These include phosphatases that remove phosphate groups from JAKs and STATs, some SOCS proteins that can competitively bind to receptor binding sites of STATs and can target JAK/STATs for proteasomal degradation, as well as protein inhibitors of activated STAT (PIAS), which prevents DNA binding and nuclear translocation of STATs [24,29]. As transcription of *SOCS* genes are regulated by STATs, this negative feedback loop provides an additional level of control over the pathway and ensures that activation of JAK/STAT signaling is transient.

While the JAK/STAT pathway appears relatively simple compared with other intracellular signaling pathways, the diversity of ligands and receptors that can activate the pathway as well as the relationship between different JAKs and STATs contribute to its complexity and the range of cellular responses. For example, STAT3 and STAT5A/B have been found to promote cancer progression while STAT1 has tumor suppressive effects [24,30].

Many studies have shown that the JAK/STAT pathway is often deregulated in cancer, including HCC. In fact, STAT3 was reported to be constitutively active in up to 60% of the HCC cases [31]. An increase in inflammatory signaling, growth factor stimulation, oxidative stress and epigenetic silencing of *SOCS* genes were some of the contributing factors for the upregulated JAK/STAT signaling [31]. Furthermore, 9% of HBV-related HCC cases contained missense mutations in JAK1, which were found to increase phosphorylation of JAK1 and STAT3, allowing cytokine-independent growth [32].

The role of *STAT3* in HCC

STAT3 is generally accepted as a bona fide oncogene in promoting HCC development. Activation of *STAT3* as a transcription factor leads to the expression of several genes which contribute to the various hallmarks of cancer, highlighting the essential role of *STAT3* in HCC (Figure 3).

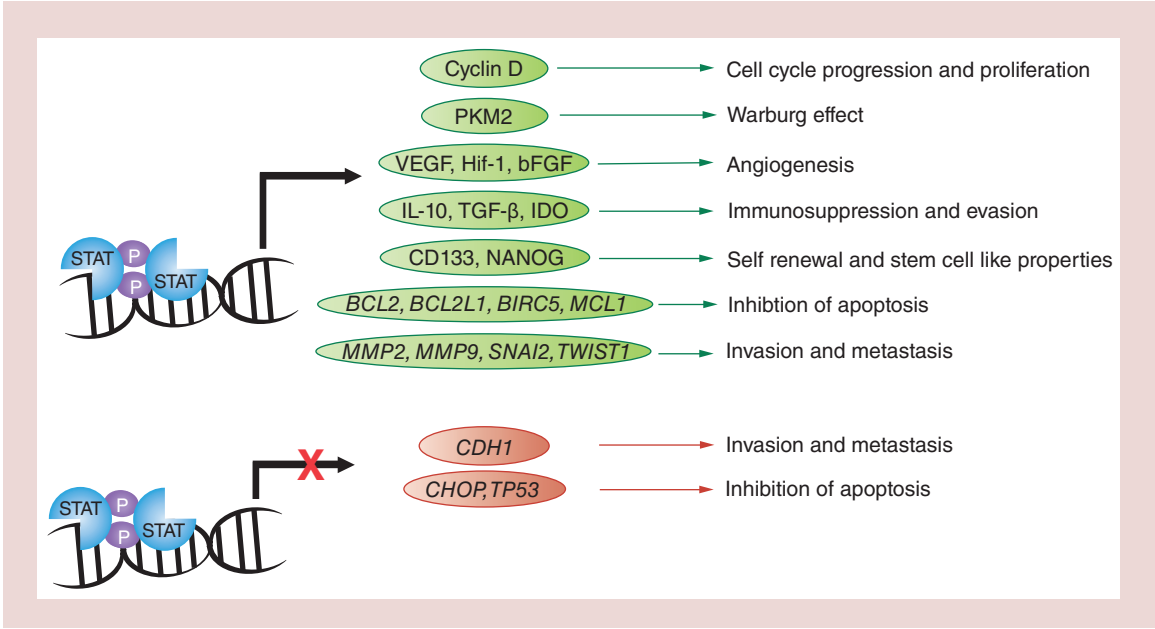

Figure 3. The role of STAT3 in hepatocellular carcinoma. The regulation of target genes and proteins by STAT3 promotes the progression of hepatocellular carcinoma by contributing to key hallmarks of tumorigenesis. Shown in green are genes and proteins which are upregulated while genes in red are inhibited by STAT3 activation.

STAT3 in survival & proliferation

The oncogenic and proliferative potential of STAT3 was first reported in 1999 [33]. Cells with constitutively activated STAT3 express higher levels of CCND1, which drives cell cycle progression from the G1 to S phase [33]. The pharmacological inhibition of the JAK2/STAT3 pathway has shown a marked downregulation of CCND1 and growth arrest at the G0/G1 phase in HCC cell lines [34]. The proliferative properties of STAT3 were also observed *in vivo*. Nude mice injected with cells harboring STAT3 clones grew tumors at the site of injection as opposed to STAT3-negative cells [33]. Similarly, the introduction of STAT3-specific short-hairpin RNA in diethylnitrosamine-induced HCC mice models failed to induce tumor development, supporting the oncogenic role of STAT3 in HCC [35]. Conversely, the inhibition of STAT3 activity via antisense oligonucleotides (ASOs) successfully mitigated tumor growth, leading to a reduction in tumor volume and doubling of survival time in orthotopically-implanted HCCLM3 mice models of HCC [36].

In addition, STAT3 is known to drive the expression of anti-apoptotic genes such as *BCL2, BCL2L1, BIRC5* and *MCL1* [37–40]. Simultaneously, STAT3 inhibits the expression of pro-apoptotic proteins such as TP53, BAX and CHOP [41,42]. Inhibition of STAT3 by JAK2 inhibitor, AG490, further induced apoptosis in HCC cell line, Hep3B, by downregulating the expression of anti-apoptotic proteins Bcl-xL and survivin [34]. Additionally, STAT3 has also been implicated in the development of sorafenib resistance in HCC cell line Huh7 by the regulation of anti-apoptotic protein, Mcl-1 [43]. The overexpression of JAK1/2 and constitutive phosphorylation of STAT3 (Tyr705) results in the nuclear localization of STAT3 and expression of Mcl-1 [43]. Knockdown of STAT3 led to a downregulation of Mcl-1 expression *in vitro*, rendering the cells sensitive to sorafenib-induced cell death. Collectively, these findings demonstrate that STAT3 promotes cell survival and drug resistance while allowing HCC cells to evade apoptosis and continue proliferating.

STAT3 in angiogenesis

STAT3 can promote angiogenesis by regulating the expression of several pro-angiogenic modulators in the tumor microenvironment. These are traditionally attributed to the bFGF, VEGF and HIF-1 axis [28,44,45]. STAT3 activation was shown to regulate the expression of Akt, a key regulator of Hif-1 expression [45]. Hif-1, together with STAT3, function as transcriptional activators of VEGF by binding to the VEGF promoter [45]. VEGF secreted by the cells can then bind to receptors on endothelial cells and stimulate the formation of new blood vessels. Conversely, inhibition of STAT3 has also been shown to downregulate the activation of the PI3K/Akt pathway and subsequent Hif-1 and

VEGF expression [44,45]. The use of anti-STAT3 ASOs can also lead to a reduction in tumor microvessel density as a result of decreased circulating VEGF and bFGF levels *in vivo* [36]. Therefore, elevated STAT3 levels facilitate tumor development by upregulating pro-angiogenic factors, thereby providing tumors with greater perfusion and promoting tumor growth.

STAT3 in immunity & inflammation

STAT3 plays a key role in regulating the inflammatory and immune environment of the tumor. STAT3 activation in tumor cells aids in evasion of immune surveillance during hepatocarcinogenesis through several mechanisms, one of which is by maintaining an activation loop with the immune cells present in the tumor microenvironment. Activation of STAT3 in tumor cells by IL-6 triggers a downstream inflammatory response, inducing the expression and secretion of STAT3-activating cytokines and chemokines such as IL-6 and IL-1b [46]. Consequently, these secreted factors activate STAT3 signaling in the surrounding stromal cells, which also synthesize and secrete the same cytokines, resulting in a paracrine tumor-stroma positive feedback or activation loop [47].

This continuous activation of STAT3 modulates the tumor immune microenvironment, ensuring that it is conducive for the tumor cell. The activation of STAT3 in regulatory dendritic cells derived from carcinoma-associated fibroblasts leads to increased secretion of IDO *in vitro* [48]. IDO impairs T-cell proliferation and response, promoting the survival of the tumor cells. Additionally, STAT3 induces the differentiation of monocytes into myeloid-derived suppressor cells *in vitro*, further impeding T-cell function and suppressing the antitumor immune response [49].

Apart from IL-6, immune cells may also respond to other interleukin and inflammatory molecules present in the microenvironment, leading to STAT3 activation. For instance, the presence of IL-4 leads to the activation of STAT3 in macrophages, inducing a polarization from the antitumoral M1 phenotype into the pro-tumorigenic M2 phenotype both *in vitro* and *in vivo* [50]. The presence of M2 macrophages in the microenvironment promotes proliferation, invasion and migration of HCC cells [50].

The tumor-promoting effects of STAT3 signaling is further established when tumor progression is inhibited upon blockade of the IL-6/STAT3 pathway, resulting in an alteration of the cytokines present in the microenvironment. Specifically, TGF-β and IL-10 levels were reduced while type I interferon expression was elevated, which reactivates natural killer cells and recovers the antitumor immune response [51].

In summary, STAT3 activation is essential in modulating the immune cells and cytokines present in the tumor microenvironment to ensure that the tumor cells can evade apoptosis and survive. Targeting STAT3 therefore offers a potential immunotherapy for HCC by suppressing the tumor immune microenvironment.

STAT3 in cancer stem cells

The activation of STAT3 has been demonstrated to correlate with cancer stem cell markers that confer stem cell-like properties to tumor cells. STAT3 activation has been shown to correlate with the self-renewing side population/CD44-positive (SP/CD44$^+$) cells in HCC [52]. CD44 is traditionally reported to maintain cell populations with cancer stem cell-like properties in HCC [53]. Inhibition of STAT3 via small molecule inhibitors significantly reduced the SP/CD44$^+$ cells *in vitro* and diminished the tumor formation capacity *in viv*o. This demonstrates the potential of targeting STAT3 in controlling the population of cells containing stem cell-like properties in HCC [52]. Additionally, STAT3 phosphorylation and activation have also been reported to regulate the expression of other cancer stem cell markers in HCC, namely CD133 and NANOG [54,55]. Inhibition of STAT3 led to a reduction in the population of cancer stem-like cells *in vitro* and impeded the tumor-initiating capacity of HCC cells *in vivo*. STAT3 has also been implicated in the maintenance of the stem cell-like population of cells in HCC via the upregulation of the Notch signaling pathway [56]. Attenuation of the IL-6/STAT3 pathway led to a deactivation of the Notch pathway, hindering the growth and invasion of HCC cells [56]. Collectively, the evidence demonstrates the role of STAT3 in maintaining the population of cells with stem cell-like and tumor initiating capacities in HCC. Thus, targeting STAT3 offers a promising strategy to reduce the population of cancer stem cells with self-renewal capabilities in HCC tumors.

STAT3 in HCC metabolism

STAT3 is implicated in the adaptation of metabolic processes in cancer cells to allow efficient generation of energy biomolecules like ATP [57]. Bi *et al.* showed that STAT3 and PKM2 can be activated and enhance the Warburg effect in HCC [58]. The Warburg effect occurs when cancerous cells transform significant amounts of glucose into

lactate regardless of oxygen availability. PKM2 is a key enzyme that regulates this process, which allows tumor cells to meet the energetic demands for expansive proliferation. Bi *et al.* observed that in transformed hepatic progenitor cells, the consumption of glucose, production of lactate and ATP levels were all decreased following the use of a small molecule inhibitor of STAT3 (Stattic) *in vitro* [58]. Additionally, this inhibition of STAT3 also decreased phospho-PKM2 expression [58]. This suggests that STAT3 regulates the expression of PKM2 and, in turn, plays a key role in altering cancer cell metabolism to meet the energetic demands of the disease. Targeting STAT3 can thus help to mitigate this aspect of HCC and reduce tumor growth.

STAT3 in invasion & metastasis

STAT3 plays a role in promoting the invasive capacities of HCC cells by regulating the expression of MMPs, such as MMP-2 and MMP-9 [36,59]. Secreted MMPs cleave the extracellular matrix in the tumor microenvironment, removing the physical barrier for cancer cells to invade into the surrounding tissue. Li *et al.* showed that anti-STAT3 ASOs reduced MMP-2 and MMP-9 expression levels *in vitro* [36]. Consequently, this inhibited lung metastasis formation and significantly prolonged survival time [36]. Zhao *et al.* also demonstrated that inhibition of STAT3 phosphorylation by CTS successfully impeded the invasion of HCC cells which was induced by peri-tumor fibroblasts [60]. Besides, STAT3 can also induce the invasiveness of HCC tumors by upregulating the expression of epithelial–mesenchymal transition proteins such as Slug and Twist [61,62]. In fact, targeting STAT3 led to a reduction in these proteins, while simultaneously increasing the expression of adhesion protein, E-cadherin, to reduce the metastatic potential of the cells both *in vitro* and *in vivo* [63].

Furthermore, STAT3 may promote the invasiveness of HCC by regulating alternative oncogenic pathways involved in metastasis. One such pathway is the PI3K/Akt2 signaling pathway, which modulates cell adhesion and invasion both *in vitro* and *in vivo* [64,65]. Zhang and colleagues demonstrated that STAT3 performs this function by regulating the expression of *AKT2* [65]. HCC cells transfected with STAT3-specific small interfering RNAs (siRNAs) induced a downregulation of *AKT2* expression along with its target genes, leading to a significant reduction in the invasive properties of the cells *in vitro* [65]. The direct crosstalk between the JAK/STAT and PI3K/Akt2 pathway further supports the role of STAT3 in promoting invasion and metastasis in HCC.

Overall, these studies demonstrate that STAT3 is a key regulator in the progression and development of HCC through its involvement in various aspects of tumorigenesis and metastasis. Thus, STAT3 offers a viable therapeutic target for treatment of HCC patients, allowing for early intervention by clinicians before the disease worsens.

The role of other STATs in HCC

Previous studies have shown that STAT1 and STAT2 exhibit antiproliferative effects in HCC both *in vitro* and *in vivo* [66]. The suppression of STAT1 activity was shown to correlate with the progression of HCC and prognosis in a set of HCC patient samples [67]. Additionally, suppression of STAT1 activity correlated with VEGF levels in HCC patients, indicating that STAT1 may exert its antitumorigenic properties by inhibiting angiogenesis [67]. Notably, the antitumorigenic properties of STAT1 correlate with the activity of STAT1 instead of the expression levels of the protein itself. In a recent study, the authors found that elevated expression of STAT1 without activation (i.e., unphosphorylated STAT1) was observed in HCC patients [68]. The presence of unphosphorylated STAT1 was able to sustain growth in HCC cell lines *in vitro* [68]. This is an important finding in guiding the development of drug targets against STAT1 as a therapeutic strategy. Specifically, it demonstrates that inducing STAT1 expression is not sufficient in treating HCC and might worsen the disease. Instead, specific activation of STAT1 serves as a more viable therapeutic strategy for HCC.

Similar to STAT1, STAT2 exhibits anticancer properties in HCC. STAT2 can exert its antitumorigenic properties in HCC by functioning as a transcription regulator of oncogenes. Testoni *et al.* showed that, in response to IFN-α induction, phosphorylated STAT2 directly binds to the the P2p73 promoter of oncogene DNp73 [69]. As a result, STAT2 recruits Ezh2 to the promoter to induce histone 3 lysine 27 methylation and hence the transcriptional repression of the oncogene [69]. The detailed antitumorigenic roles of STAT1 and STAT2 in HCC, however, still remain relatively unclear.

While the detailed role and function of STAT4 in HCC is not well established, several publications have demonstrated that STAT4 likely functions as a tumor suppressor in HCC. In these studies, the authors reported a significantly lower expression level of STAT4 in HCC tumors as compared with the normal tissues [70,71]. Furthermore, the knockdown of STAT4 via siRNAs in these two independent studies led to enhanced proliferation of HCC cell lines *in vitro*. In addition, several studies investigating polymorphisms in HCC patients have identified

a specific STAT4 polymorphism (rs7574865) which increases the risk of HBV-related HCC in several cohorts, including Chinese, Thai, Korean, Vietnamese and Caucasian. This specific rs7574865 STAT4 variant also showed a corresponding reduction in *STAT4* mRNA expression levels [72–76]. However, there are still no conclusive studies detailing the exact role of the variant.

The distinct functions of both isoforms of STAT5 (STAT5a and STAT5b) in HCC is still unclear and shows context specificity. Upregulation of STAT5 has been observed in HCC patients, suggesting that STAT5 exhibits pro-oncogenic properties [77]. Specifically, one group identified that STAT5b, and not STAT5a, was a driver of epithelial–mesenchymal transition in HBV-dependent HCC *in vitro* [78]. Several mouse models of HCC, however, have suggested that STAT5 exhibits hepatoprotective properties instead [66,79–81]. One group showed that STAT5 acts as a tumor suppressor in the context of hyperactive growth hormone signaling. In the study, the authors demonstrated that mice with hyperactive growth hormone signaling and a synthetic loss of STAT5 rapidly developed HCC as a result of increased compensatory STAT3 activation [79]. In a second study, Yu *et al.* demonstrated that in CCl_4-induced HCC mouse models, STAT5 functions as a tumor suppressor by upregulating the expression of *CDKN2B* and *CDKN1A* [81]. Ablation of STAT5 *in vivo* led to a reduction in the $p15^{INK4B}$ protein levels, a compensatory activation of STAT3 and tumor progression [81]. Kaltenecker *et al.,* however, did not witness a more aggressive HCC phenotype when STAT5 was lost in their diethylnitrosamine-induced mice models [82]. Taking the evidence together, they suggest that the function of STAT5 is extremely complex and context-dependent, thereby calling for deeper investigation into the role of STAT5 in HCC in humans.

Targeting the JAK/STAT pathway for HCC treatment

Different JAK/STAT inhibitors are already being studied for their clinical relevance in various cancers, including HCC. Two main classes of molecules that have been used are small molecule inhibitors and siRNAs (Table 1).

Targeting JAKs

As JAKs function upstream of STATs along the signaling axis, it acts as a feasible target to inhibit the downstream effects of the JAK/STAT pathway. Currently, WP1066, pacritinib, cryptotanishinone and ruxolitinib are common JAK inhibitors being studied for their relevance in human diseases; however, these compounds are still in preclinical stages for HCC treatment.

Studies into brain and bladder cancers have demonstrated the efficacy of WP1066, which inhibits JAK2. In malignant glioma cells, WP1066 downregulated downstream targets of STAT3 such as Bcl-xL, Mcl-1 and c-myc. The drug also selectively activated BAX, induced apoptosis and downregulated several anti-apoptotic proteins. Thus, these findings indicate a strong correlation between WP1066 and programmed cell death. Currently, WP1066 is being investigated for brain metastasis in clinical trials (NCT01904123) [83,86]. Similarly, another study by Tsujita *et al.* showed that WP1066 demonstrated efficacy by promoting apoptosis of bladder cancer cells [84]. In HCC, WP1066 has been shown to inhibit MMPs and neutralize the activity of UCK2, which reduced the migration and invasion abilities of HCC cell lines [85]. Collectively, these insights demonstrate the potential of WP1066 as a treatment approach in HCC.

Another potent and selective JAK2 inhibitor is pacritinib, which was observed to have clinical efficacy for myelofibrosis patients when compared with the current best available treatment in a randomized Phase III trial (NCT02055781) [87]. Overall, the outcome showed improved total symptom score reduction and effective spleen volume reduction in pacritinib-treated patients compared with the best available treatment. Pacritinib was also well tolerated and adverse events were uncommon, with patients generally presenting mild gastrointestinal toxic effects. In another study, Jensen and colleagues used patient-derived brain tumor-initiating cells and demonstrated that pacritinib was able to reduce cell viability in these cells with satisfactory results [86]. Moreover, the team determined the compatibility of pacritinib in combination with temozolomide, the current standard of care chemotherapy for glioblastoma multiforme. This combination in mice xenografts led to an improvement in median survival, from 52 to 62.5 days, and reduced tumor growth. Thus, this study demonstrates the potential of pacritinib, as the JAK inhibitor could prolong survival periods in clinically relevant models. In HCC, pacritinib was found to reduce liver fibrosis in mouse models that mimic clinical HCC development and progression from hepatic steatosis [88]. High levels of CK-18, which predicts for increased fibrosis, was also effectively reduced in these mice upon treatment with pacritinib [88]. Given that liver fibrosis occurs in most patients with HCC, the antifibrotic effects of pacritinib observed in relevant disease models offer promising clinical applications for HCC treatment.

Table 1. Clinical status of Jak/STAT inhibitors.

Classification: small molecule inhibitors	Indication	Target	Clinical status	Findings/results	Ref.
WP1066	Bladder cancer Malignant glioma Metastatic melanoma HCC	JAK2	Phase I (NCT01904123) (Pre-clinical for HCC)	*In vitro* studies: in bladder and brain cancer cells, pre-clinical studies show promotion of apoptosis. Awaiting results for metastatic melanoma and malignant glioma in clinical trials (NCT01904123). In HCC: WP1066 was shown to inhibit MMPs and reduce migration and invasion of HCC cancer cells.	[83–85]
Pacritinib	Malignant glioma Myelofibrosis HCC	JAK2	Phase III (NCT02055781) (Pre-clinical for HCC)	*In vitro* studies: pacritinib decreased BTIC viability and sphere-forming potential. Improved response to TMZ in TMZ-resistant BTICs was also observed. *In vivo* studies: in orthotopically xenografted mice, pacritinib combined with TMZ showed penetration of the blood-brain barrier and led to overall median survival improvement. Phase III: for myelofibrosis and thrombocytopenia patients including those prior anti-Jak therapy, the twice-daily dosing of pacritinib was more effective than the best available treatment for reducing splenomegaly and symptoms. In HCC: *in vivo* studies showed fibrosis biomarker CK18 was effectively reduced by pacritinib. Fibrotic areas were also reduced in the mouse liver.	[86–88]
CTS	Esophageal cancer HCC	JAK2, STAT3	Pre-clinical	*In vitro* and *in vivo* studies: migration and tumor growth of esophageal cancer cells was impeded with CTS. Inhibition of cell growth in mice xenografts was also observed without significant effect on body weight. In HCC: CTS was shown to promote apoptosis and immune response *in vitro* and *in vivo*. CTS also helped convert immune cells to the tumor suppressive M1 phenotype.	[60,89,90]
Ruxolitinib (INCB018424)	Leukemias HCC	JAK1/2	Approved Phase II (NCT00674479) (Pre-clinical for HCC)	Approved for myelofibrosis, polycythemia vera, graft-vs-host disease. Phase II: modest antileukemia activity and an acceptable toxicity profile were seen in refractory leukemias. This includes post myeloproliferative neoplasm acute myeloid leukemia patients. In HCC (preclinical): Ruxolitinib was shown to inhibit colony-forming abilities and cell proliferation.	[91,92]
Stattic	NPC HCC	STAT3	Pre-clinical	*In vitro* studies: reduced growth and increased apoptosis of NPC was observed with Stattic. The drug also sensitized NPC to cisplatin and ionizing radiation. In HCC: Stattic-attenuated radiotherapy and reduced cancer functions such as invasiveness, survival and proliferation.	[93,94]
OPB-111077	Advanced HCC	STAT3	Phase I (NCT01942083)	The drug was compatible with advanced HCC patients that failed sorafenib therapy. Limited preliminary efficacy outcomes were shown.	[95]
OPB-31121	Advanced cancer Solid tumor HCC	STAT3	Phase I (NCT00657176) PhaseI/II (NCT01406574)	(NCT00657176): OPB-31121 was relatively well tolerated and has preliminary antitumor activity in solid tumors. In HCC (NCT01406574): limited survival benefits and insufficient antitumor activity were shown	[96,97]
Napabucasin (BBI608)	Gastric cancer HCC	STAT3	+ Paclitaxel: Phase I (JapicCTI-142420) Phase III (NCT02178956) + Sorafenib: Phase Ib/II (NCT02279719)	(JapicCTI-142420): for Japanese patients with gastric cancer, the combination of napabucasin with paclitaxel was tolerated. No dose-limiting toxicities were observed and two patients reported partial response, stable disease and progressive disease each. Trial is ongoing for NCT02178956. In HCC (NCT02279719): recommended Phase II dose was determined for napabucasin and safely combined with sorafenib at full dose. Encouraging antitumor activity seen in HCC patients with no prior systemic chemotherapy.	[98,99]
AZD9150	Advanced cancers DLBCL Lymphoma HCC	STAT3	Phase I/II (NCT01563302) Phase I/Ib (NCT01839604))	(NCT01563302): in a subset of heavily pretreated DLBCL patients, AZD9150 was well tolerated and efficacious. In HCC (NCT01839604): maximum tolerated dose for AZD9150 was determined with preliminary activity and few serious adverse effects.	[100,101]

BTIC: Brain tumor-initiating cell; CTS: Cryptotanshinone; DLBCL: Diffuse large B-cell lymphoma; HCC: Hepatocellular carcinoma; NPC: Nasopharyngeal carcinoma; TMZ: Temozolomide.

CTS is a plant-based quinone extracted from the root of *Salvia miltiorrhiza Bunge* that has inhibitory effects on the JAK/STAT pathway. In preclinical studies, CTS was shown to induce apoptosis, inhibit proliferation and reduce the migration of esophageal squamous cell carcinoma. In mice, tumor growth was effectively reduced with CTS treatment, with minimal effects on body weight, indicative of its low toxicity profile. Therefore, CTS has

been shown to be a viable alternative for the treatment of esophageal squamous cell carcinoma [89]. In HCC, CTS treatment was found to inhibit the proliferation of mouse hepatoma cells by promoting cell apoptosis via JAK/STAT signaling. Furthermore, CTS was shown to promote immune response *in vivo* and aid in the conversion of macrophages to the M1 phenotype *in vitro*, allowing for increased proinflammatory and antitumor properties [90].

Ruxolitinib, a small molecule inhibitor of JAK1 and JAK2, was the first JAK inhibitor to be approved by the FDA (for primary myelofibrosis). Recently, a Phase II study of ruxolitinib in relapsed/refractory leukemia (NCT00674479) demonstrated satisfactory results, with limited grade 3 or higher toxicity [91]. Significant response was observed in 17% of postmyeloproliferative neoplasm acute myeloid leukemia patients, with complete remission in two patients. While ruxolitinib has been widely studied for the treatment of blood malignancies, studies in HCC are still preclinical. Ruxolitinib was shown to inhibit cell proliferation and colony-forming abilities in HCC cell lines [92]. Additionally, using HCC patient-derived xenograft models, tumors with *JAK1* mutations ($JAK1^{S703I}$) were observed to have increased sensitivity toward ruxolitinib as compared with other JAK1 mutant or wild-type tumors [102].

Targeting STAT3

Besides indirectly inhibiting STAT3 via JAKs, STAT3 can also be directly inhibited using small molecule compounds such as stattic, OPB-111077, OPB-31121, napabucasin or AZD9150, an siRNA.

Stattic has been shown to inhibit the activation, dimerization and translocation of STAT3 independent of its phosphorylation status [103]. In nasopharyngeal carcinoma (NPC) cell lines, Stattic promotes antitumor effects by decreasing the expression of STAT3-mediated CCND1 [93]. Furthermore, Stattic was shown to induce apoptosis and inhibit cell viability, effectively impeding cancer growth *in vitro* [93]. As a combination therapy, Stattic was shown to synergize well with other treatments such as cisplatin, as evidenced by the lowered IC_{50} values in NPC cells, demonstrating increased drug potency [93]. In HCC cell lines, Stattic promoted apoptosis induced by radiotherapy and reduced tumor cell survival and invasiveness in a dose-dependent manner [94].

In a Phase I study by Yoo *et al.*, OPB-111077 was administered to sorafenib-refractory HCC patients to examine toxicity and safety profiles [95]. The drug was well tolerated overall, with limited patients experiencing dose-limiting toxicities and no treatment-related deaths reported. Unfortunately, preliminary outcomes showed limited efficacy, with zero cases of complete or partial response and a median progression-free survival of 1.4 months. Nevertheless, further investigations of OPB-111077 for combination therapy can be considered due to its acceptable safety profile.

Another STAT3 inhibitor is OPB-31121 which has been evaluated in a Phase I study for advanced solid tumors (NCT00657176) [96]. Common adverse events observed were gastrointestinal and included nausea, vomiting and diarrhea. Evaluable dose-limiting toxicities included grade 3 diarrhea and grade 3 vomiting. Overall, 800 mg/day was determined as the maximum tolerated dose. Stable disease was seen in eight patients, while disease progression was present in ten patients. Furthermore, OPB-31121 showed tumor shrinkage in one colon cancer and one rectal cancer patient. Hence, although the side effects are not ideal, the study demonstrates preliminary efficacy of OPB-31121 in advanced solid tumor patients. However, in a Phase I study by Okusaka *et al.* (NCT01406574), OPB-31121 showed poor antitumor efficacy in advanced HCC patients [97]. Despite six out of 25 patients having stable disease ($\geq$8 weeks), the toxic side effects associated with the peripheral nervous system limited potential long-term usage of the drug.

Napabucasin is a STAT3 inhibitor that has been found to have preliminary clinical efficacy. In a clinical trial of napabucasin combined with paclitaxel (JapicCTI-142420), the two drugs were shown to be well tolerated in Japanese patients with gastric cancer [98]. Common adverse effects reported were generally mild and gastrointestinal, but concurrent administration with loperamide was able to control these negative effects. Napabucasin was found to have a satisfactory safety profile. Two patients in the study demonstrated preliminary signs of clinical activity in which partial response was achieved. In one of these patients, even after paclitaxel was discontinued at cycle 7, partial response was maintained until cycle 21 with napabucasin treatment alone. Collectively, this study demonstrates that drug combinations with napabucasin are a viable treatment approach with potential survival benefits and hence a Phase III trial is ongoing for this combination in gastric and gastroesophageal junction cancers (NCT02178956) [98]. In preclinical studies of HCC, napabucasin promoted apoptosis *in vitro* and suppressed tumor growth in orthotopic mouse models [104]. Using another orthotopic HCC resection mouse model, napabucasin treatment was also observed to decrease the incidence of recurrence after surgery (hepatectomy), which is likely mediated by inhibition of the IL-11/STAT3 signaling axis [104]. In addition, AFP and proliferating cell nuclear

antigen levels were also reduced after treatment with napabucasin. Hence, there is evidence that targeting STAT3 signaling can reduce tumor growth and mitigate the risk of recurrence. Currently, napabucasin is being evaluated in a phase Ib/II clinical trial in combination with sorafenib for HCC (NCT02279719).

In contrast to small molecule inhibitors, AZD9150 is a siRNA that targets STAT3. In a Phase Ib study by Reilley *et al.*, diffuse large B-cell lymphoma patients were treated with AZD9150 and the drug was well tolerated (NCT01563302) [100]. Drug-related side effects such as fatigue, transaminitis and thrombocytopenia were commonly observed; however, no patient withdrew treatment due to drug-related toxicities. Complete and partial responses were observed in two patients each with a median duration of response of 10.7 months for the complete response patients. Altogether, AZD9150 showed clinically meaningful antitumor activity and can be considered as a safe therapy for diffuse large B-cell lymphoma. In HCC, a Phase I/Ib study was performed with AZD9150 to evaluate its efficacy and safety profiles (NCT01839604) [101]. The study showed that AZD9150 was well tolerated with mild and a few serious adverse events. However, further studies are needed to elucidate its clinical efficacy.

Overall, these studies demonstrate the potential of targeting the JAK/STAT pathway in HCC. While clinical investigations for these inhibitors are still in early stages for HCC, the beneficial effects observed in other tumor types provide indications of possible clinical efficacy for HCC as well.

Future perspective

Activation of JAK/STAT signaling is a widely reported phenomenon in many cancers, including HCC. The multiple cellular effects of JAK/STAT signaling and its relationship with other signaling pathways have been shown to contribute to many key hallmarks of cancer development and progression. Thus, therapeutic targeting of activated JAK/STAT pathway is a rational approach in tumors with such aberrant signaling. In fact, the numerous clinical trials and studies on JAK/STAT inhibitors demonstrate the potential and efficacy of these compounds in mitigating various cancers. Nevertheless, in the case of HCC, clinical investigations into these compounds are still in the early stages, with limited benefits observed. As many of these studies in HCC were done using JAK/STAT inhibitors as monotherapy, perhaps these compounds could be applied in a combinatorial therapy setting. With its role in maintaining cancer stem-like cells, targeted inhibition of JAK/STAT could be investigated as an adjuvant therapy for HCC, which may help to suppress or eradicate the subpopulation of cells with tumor-propagating properties. Furthermore, as these cells often mediate chemoresistance in tumors, the use of JAK/STAT inhibitors could help mitigate this, thus reducing the risk of drug resistance and disease recurrence, two major setbacks in the current treatment landscape of HCC. Besides, given the highly heterogenous nature of HCC, it is likely that the response toward JAK/STAT inhibitors among patients would be varied as well, as seen in the case of increased ruxolitinib sensitivity in HCC patient-derived xenograft tumors with specific *JAK1* mutations. Thus, it would be interesting to see if these mutations that correlate with treatment efficacy can be applied toward stratifying HCC patients in clinical trials to achieve better outcomes and survival. Overall, the JAK/STAT pathway is a promising therapeutic target for HCC, although further investigations are needed to fully understand the molecular mechanisms and side effects to improve clinical outcomes and possible personalized treatments.

Author contributions

JJH Tang, DKH Thng, JJ Lim and TB. Toh were major contributors in the writing of the manuscript. TB. Toh designed, revised and edited the manuscript. All authors read and approved the final manuscript.

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Executive summary

- Hepatocellular carcinoma (HCC) is a highly challenging disease to treat, due to factors such as lack of effective treatments, high rate of recurrence, underlying liver dysfunction, drug resistance and heterogeneous tumor background.
- Treatment options for HCC include surgical resection, liver transplantation, transarterial chemo- or radio-embolization and systemic drug therapy using small molecule inhibitors such as sorafenib, lenvatinib, regorafenib and cabozantinib.
- Many intracellular signaling pathways contribute to hepatocarcinogenesis, including the JAK/STAT pathway, which has normal roles in regulating cell proliferation, survival and differentiation. However, deregulation of JAK/STAT signaling is observed in many cancers and contributes to various oncogenic effects.
- In HCC, aberrant activation of JAK/STAT pathway promotes tumor growth, angiogenesis, invasion and metastasis. JAK/STAT signaling is also implicated in maintenance of cancer stem cells with tumor-propagating abilities in HCC as well as creation of an immunosuppressive microenvironment.
- Due to the oncogenic role of JAK/STAT activation, especially in the context of STAT3 dysregulation, targeting this pathway represents an attractive/feasible approach for the treatment of HCC. In fact, various small molecule inhibitors and RNA therapies that target JAKs or STATs have been developed and tested for efficacy against tumor cells.
- While many of the JAK/STAT-targeting compounds have shown clear antitumor effects on tumor growth and development in preclinical models of HCC, clinical investigations of these compounds for HCC are still limited. Nevertheless, the promising results of clinical trials in other cancer types highlight the potential of inhibiting the JAK/STAT pathway as an effective treatment for HCC.

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