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REVIEW

The Eph/Ephrin System in Hepatocellular Carcinoma: Functional Roles and Potential Therapeutic Targets

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ABSTRACT

Liver cancer is ranked as the second leading cause of cancer-related death across the world, and hepatocellular carcinoma (HCC) comprises nearly 90% of all primary liver cancer cases. However, until now, the pathological mechanisms underlying HCC oncogenesis and development remains largely unclear. Eph receptors and their ephrin ligands (Eph/Ephrin system) are involved in various physiological process, such as embryogenesis, neuronal migration, immune response and vascular development. Recently, emerging evidence has showed that the Eph/Ephrin system also plays an essential role in initiation and development of multiple types of cancer, including liver cancer. The Eph/Ephrin system has been reported to contribute to cellular proliferation, apoptosis, migration, invasion, angiogenesis, stemness and drug/radiotherapy resistance in HCC. Thus, the aim of this review is to summarize the effects and underlying mechanisms of every member of this system in HCC, with special focus on assessing their therapeutic potential as drug targets.

KEYWORDS

Hepatocellular carcinoma; Eph receptor; Ephrin ligand; molecular mechanism; therapeutic target

1 Introduction

Liver cancer is ranked as the second leading cause of cancer-related death across the world [1]. As the most common type of liver cancer, hepatocellular carcinoma (HCC) comprises nearly 90% of all primary liver cancer cases with a prevalence of 10.1 cases in 100000 worldwide [2,3]. The incidence of HCC is associated with geography and household income [4]. The majority of HCC occur in eastern Asia and Africa. At present, Hepatitis B virus (HBV) and hepatitis C virus (HCV) remain the most dominant etiologic factors for HCC, but effective prevention and treatment will decrease incidence of HBV/HCV-associated HCC in the coming years [5]. Recently, non-alcoholic fatty liver disease (NAFLD) is emerging as an important cause of HCC with the most rapidly increasing incidence [6]. In addition, excessive alcohol consumption and aflatoxin are also important risk factors of HCC [5]. Patients with HCC have usually developed advanced-staged disease at the time of diagnosis, for whom targeted therapy is an important strategy to control tumor progression. Although tyrosine kinase inhibitors (TKIs) have been recommended for advanced HCC patients, recent studies have shown that a considerable proportion of HCC patients are less responsive to TKIs therapy [7,8]. Therefore, a more profound and comprehensive

understanding of molecular mechanism underlying biological process of tumor, such as proliferation, invasion, migration, angiogenesis and drug resistance, will serve to establish more effective diagnostic and treatment approaches of HCC.

Cancer stem cells (CSCs) are thought as drivers of establishment and growth of HCC, and associated with aggressiveness and therapy resistance of tumors [9]. CSCs arise from either de-differentiation of mature cells or transformation of adult tissue resident stem cells [10]. Chronic inflammation plays a prominent role in inducing mutation and dedifferentiation of hepatocytes during tumorigenesis and development of HCC [11]. Liver cirrhosis caused by chronic hepatitis was founded to be present in most HCC cases (80%∼90%) and exacerbated impairment of liver function [12]. Studies have demonstrated that some cytokine signaling and immune-related pathways including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), signal transducer and activator of transcription 3 (STAT3), and nuclear factor kappa B (NF-κB) pathways are involved in chronic inflammation-related hepatocarcinogenesis [13–15]. However, the concrete mechanism has not yet been illuminated. In the past few years, dysregulation of erythropoietin-producing human hepatocellular receptors (Ephs) and their corresponding ligands ephrins was founded in multiple types of cancer including HCC and was associated with tumor initiation and/or progression [16–18]. In this review, we summarized the roles of Ephs and ephrins in HCC, and assessed therapeutic potential of targeting these molecules.

2 The Structure and Signaling of Eph/Ephrin

As the largest known subfamily of receptor tyrosine kinases (RTK), Eph receptors are classified into two categories according to their structure similarity and DNA sequence homology: EphA receptors (EphA1-8, EphA10) and EphB receptors (EphB1-4, EphB6). Generally, the EphA receptors promiscuously bind to ephrinA ligands (ephrinA1-5), while EphB receptors bind to ephrinB ligands (ephrinB1-3). However, there are some exceptions from the general principle (such as EphA4 binding to ephrinB2 and EphB2 binding to ephrinA5) [19,20].

The Eph receptors and ephrins are ubiquitously expressed on cell membrane in a variety of human cell types. The extracellular domain of Eph receptors consists of two fibronectin type III regions, a cysteine rich region and a globular domain which can bind to corresponding ephrins ligands. The intracellular domain is composed of a conserved tyrosine kinase domain, a sterile alpha motif (SAM) domain and a PDZ motif. The ephrinAs are anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) linker, while the ephrinBs have a transmembrane domain and an intracellular PDZ motif. Typically, the ligand binding activation of Eph receptors is dependent on cell-cell interaction between adjacent plasma membranes. Subsequently, bi-directional signals are triggered between the two cells (Fig. 1). In forward signaling, after ephrin ligands bind to an Eph receptor, intracellular tyrosine residues of Eph receptor are auto-phosphorylated, and some adaptor proteins (such as including Ras-GTPase-activating protein, JAK2 and PI3K) are recruited to the receptor to initiate downstream signaling cascades into the cell [21,22]. Due to lack of intracellular domain, reverse signaling of ephrinAs is mediated by co-receptors including Ret receptor tyrosine kinase, tropomyosin receptor kinase B and p75 neurotrophin receptor [23]. In contrast, as ephrinBs also contain an intracellular PDZ domain, the downstream pathways of ephrinBs reverse signaling are similar to Eph forward signaling [22]. Intriguingly, recent studies indicated a soluble ephrins ligands-mediated non-classical mechanism via autocrine and/or paracrine pathways in some pathological conditions including cancer [24–26]. Ephs-ephrins signaling mediates a variety of cellular processes including cell migration, adhesion, proliferation, and differentiation [27]. Furthermore, it is also involved in immune response and angiogenesis [28,29], which are closely related to cancer development.

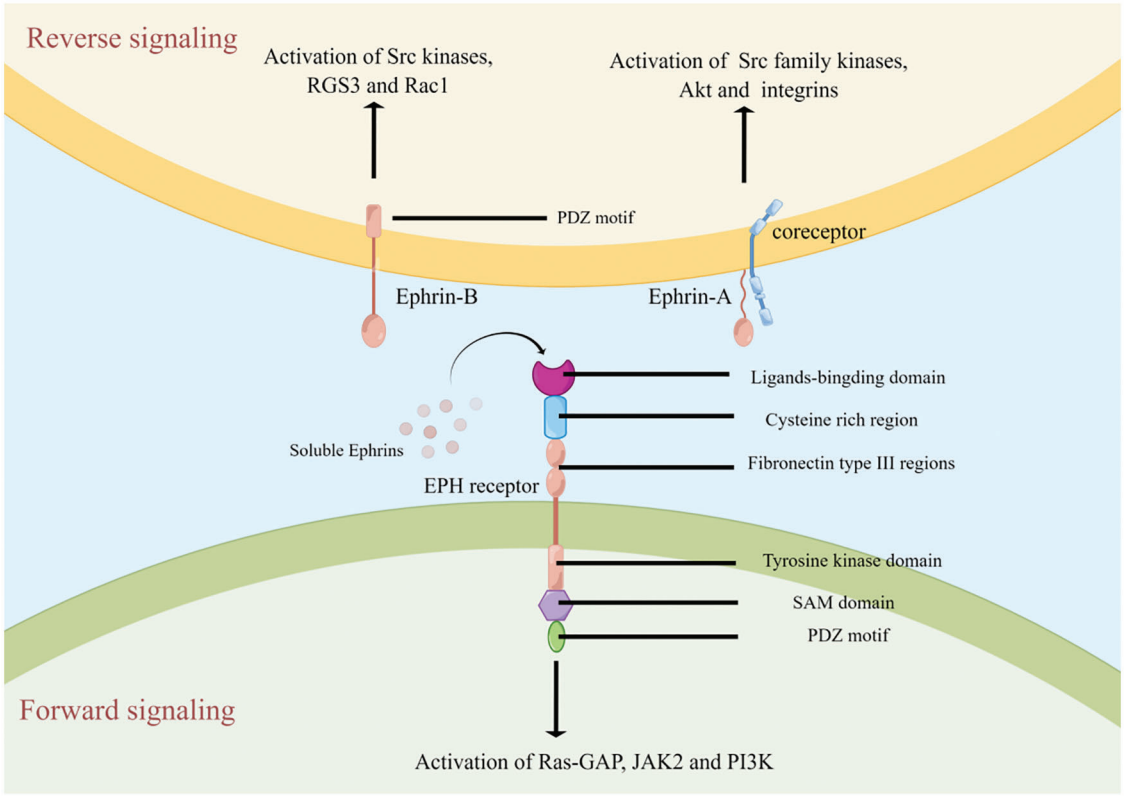

Figure 1: The structure and signaling of Eph/Ephrin system

After binding of ephrin ligands (membrane-anchored or unanchored), Eph receptors are auto-phosphorylated to activate downstream signaling including Ras-GTPase-activating protein (Ras-GAP), JAK2 and PI3K. On the other hand, the interaction between Eph receptors and ephrin ligands can also trigger reverse signaling via ephrinAs-coreceptors or ephrinBs.

3 Roles of Eph/Ephrin in HCC

Up to date, 9 of Ephs (EphA1-5, EphA7, EphB1-2 and EphB6) and 6 of ephrins (ephrinA1-5 and ephrinB1) have been investigated in HCC. In general, Eph/Ephrin system plays a pro-tumorigenic role in HCC, expect for EphA4 and ephrinA5 which exhibit an anti-tumor effect. The system has been confirmed to be involved in regulation of cellular proliferation, migration, invasion, apoptosis, angiogenesis, maintenance of stemness and drug/radiotherapy resistance in HCC (Fig. 2).

During establishment and development of HCC, each subtype of Eph/Ephrin system has a distinct role. Subtypes marked with red font promote tumorigenesis, while subtypes marked with green font suppress tumorigenesis.

3.1 Eph Receptors in HCC

EphA1

As the first discovered subtype in Eph/ephrin system, EphA1 was identified in an erythropoietin-producing human HCC cell line (ETL-1) and was deemed to be probably associated with some neoplastic process in 1987 [30]. Later, Iida et al. showed that EphA1 was overexpressed in human HCC tissues producing AFP and HCC cell lines, and that the activation of EphA1 stimulated by ephrinA1-fc

promoted proliferation of HLE cells by suppressing p21 expression [31]. Furthermore, stable knockdown of EphA1 in Huh-7 cells decreased proliferation, motility and invasion capability *in vitro* and reduced tumor growth as well as microvascular density *in vivo* [32]. The loss of EphA1 also reduced the expression and MMP2, MMP9 and VEGF, which contributed to angiogenesis and development of HCC [32,33]. A recent study verified that EphA1 in HCC cells could activate the SDF-1/CXCR4 axis by PI3K and mTOR pathways and enhanced the chemotaxis of endothelial progenitor cells (EPCs) to HCC, which in turn promoted tumor neovascularization [34].

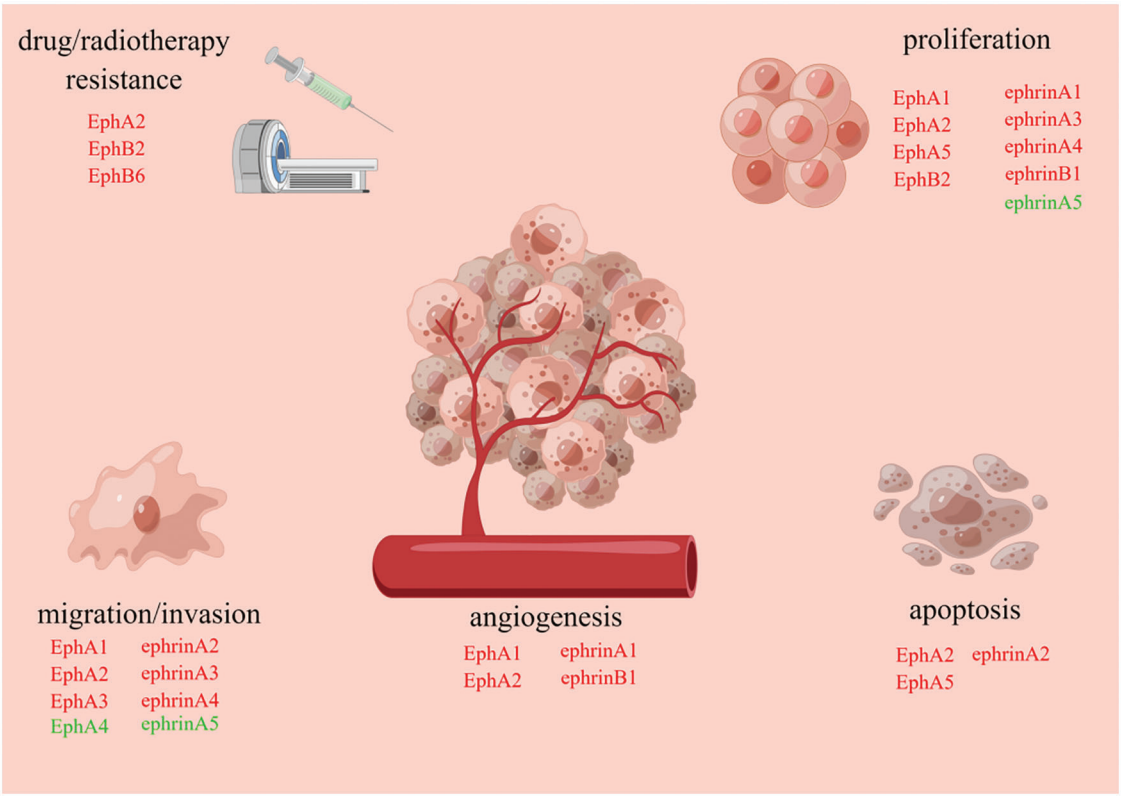

Figure 2: Eph/Ephrin system is involved in regulation of cellular proliferation, apoptosis, angiogenesis, migration, invasion and drug/radiotherapy resistance

EphA2

Multiple clinical researches have demonstrated that EphA2 was overexpressed in HCC and was associated with dedifferentiation, TNM stage, portal vein invasion, microvascular density and rate of recurrence [35–37]. Mechanistically, EphA2 drove HCC tumor initiation and progression via AKT and JAK1/STAT3 signaling [38]. In HBV-associated HCC, EphA2 also promoted HBV replication and tumor cell proliferation via p38MAPK and ERK1/2 pathways [39]. Besides, there is emerging evidence that EphA2 can enhance tumor stemness of HCC in a hypoxic microenvironment which is formed due to fast-growing nature of tumor and insufficient vasculatures [40].

Radiotherapy is an effective strategy for cancer and can elevate local control rates in patients with unresectable HCC [41]. However, resistance to radiotherapy is common [42]. The resistance to radiotherapy in HCC seems to correlate with overexpression of EphA2. In HCC cells, up-regulation of EphA2 attenuated radio-sensitivity via p38MAPK pathway, with a significant increase in cell viability

and healing capacity as well as a decline in apoptosis rate after low-dose ionizing radiation, while knockdown of EphA2 reversed the above effects [43,44]. In addition, EphA2 was also involved in paclitaxel resistance in HCC cells [45].

EphA3

Cheng et al. reported that expression of EphA3 was positively correlated with tumor grade, tumor size and TNM stage in HCC [46]. In line with this finding, the downregulation of EphA3 markedly dampened the invasive capacity of HCC cells by decreasing expression of VEGF *in vitro* [46]. Recently, some novel mutations of EhpA3, including one D219V missense mutation in the extracellular domain and two alterations in SAM domain, were detected in HCC tissues. These mutations may participate in progression of HCC [47].

EphA4

Differing from other Eph receptors, EphA4 appears to play a tumor suppressive role in HCC. Decreased EphA4 expression was observed in HCC compared adjacent non-tumor liver tissues [48]. Overexpression of EphA4 inhibited invasion and migration by way blocking the epithelial-mesenchymal transition (EMT) process in QGY-7703 cells [48,49]. This, in fact, is not surprising because activation of EphA4 can inhibit integrin signaling pathways which is essential in invasion and metastasis of tumor [50,51].

EphA5

Wang et al. observed that p-EphA5 (activated form of EphA5) was abundant in HCC tissues and HCC cell lines [52]. The activation of EphA5 is required for tumor growth and is highly associated with worse prognosis in patients with HCC. Furthermore, EphA5 was recognized as one of client proteins of Hsp90, which can help oncoproteins to avoid degradation and misfolding in HCC cells [52,53].

EphA7

The mRNA level of EphA7 was founded to be up-regulated 26-fold in HCC compared with healthy liver tissue [54]. Besides, the staining for EphA7 was clearly strong and intense in blood vessels. Similarly, expression of EPHA7 was positively related to microvascular density of tumor [55]. This suggested EphA7 may exert a pro-angiogenic signal in HCC, although further validation is still needed.

EphB1

Currently, very few studies explored the roles of EphB1 in HCC. Kim et al. identified seven polymorphisms of the EphB1 gene (rs1502174, rs9877457rs, 11929692, rs6766459, rs7644369, rs6776570 and rs3821502) which may be associated with susceptibility to HCC in the South Korean population [56]. However, the effects of EphB1 on initiation and development of HCC remain to be elucidated.

EphB2

As mentioned above, a stepwise increase of EphB2 expression was observed from normal liver to fibrotic liver to HCC tissue, and the overexpression was associated with worse prognosis in HCC patients [54,57,58]. *In vivo*, knockout of EphB2 attenuated tumor growth of HCC. Furthermore, RNA-sequencing analysis revealed that EphB2 is the most significantly upregulated kinases in sorafenib-resistant patient-derived tumor xenografts (PDX) compared with their corresponding mock controls, which suggested that EphB2 was related to drug resistance. High expression of EphB2 was also founded to enhance the stemness of HCC cells. Mechanistically, EphB2 activated AKT/GSK3β/β-catenin signaling pathway by physically interacting with and phosphorylating SRC. In turn, β-catenin/TCF1 pathway initiated transcription of EphB2 gene, which forms a TCF1/EphB2/β-catenin positive feedback loop finally [58].

EphB6

Pharmacogenomic data analysis uncovered that EphB6 mutations (mainly nonsense mutations and a missense mutation, Q926R) were involved in paclitaxel resistance in cancer [45]. Q926R mutation reduced the flexibility of the SAM domain, thereby inhibiting degradation of EphA2 by suppressing recruitment of c-Cbl ubiquitin E3 ligase. Subsequently, EphA2 promoted JNK-mediated expression of cadherin 11 (CDH11) and activated RhoA/FAK signaling pathway, which in turn induced cell adhesion-mediated paclitaxel resistance in HCC cells.

3.2 Ephrins in HCC

EphrinA1

As the ligand of EphA1 and EphA2, ephrinA1 expression was elevated in cirrhotic tissues and HCC tissues, and was significantly associated with AFP expression and prognosis of patients [31,35,59]. In HLE cells, up-regulation of ephrinA1 promoted the cellular proliferation. Furthermore, ephrinA1 regulating tumor development by inducing expression of genes related to the cell cycle, cell-cell interactions and angiogenesis. Notably, ephrinA1 is detectable in the serum of patients with HCC, suggesting it may be a useful marker for the diagnosis of HCC and a predictor for outcome of patients [35].

EphrinA2

Feng et al. described a significant increase of ephrinA2 expression in both cell lines and clinical samples of HCC, especially in the tumors with portal veins invasion [60]. Overexpression of EphrinA2 in HCC cells significantly enhanced oncogenic effect *in vivo* tumorigenicity, while down-regulation of this gene inhibited tumor growth. Further exploration revealed that the reduced Rac1/Akt/NF-κB pathway-related apoptosis, rather than enhanced proliferation, was responsible for ephrinA2-induced tumorigenesis.

EphrinA3

Hypoxia regions within tumors have been demonstrated to be associated with poor prognosis of patient and are now recognized to be involved in maintenance of stemness, metabolism, drug resistance and metastasis in various tumors [61–64]. Hypoxia inducible factor 1 alpha (HIF-1α) is one of key transcription factors that help tumors to make adaptive changes for cellular survival under hypoxia. Hypoxic-induced HIF-1α can directly promote expression of ephrinA3 to active EphA2 in HCC cells. And then, the activated ephrinA3/EphA2 axis enhanced the abilities of self-renewal, proliferation and migration in HCC by regulating cellular metabolic plasticity via promoting the maturation of SREBP1 and expression of ACLY [40].

EphrinA4

EphrinA4 was founded to be highly expressed in HCC samples and associated with poor overall survival and progression-free survival [65]. High expression of ephrinA4 promoted the cellular migration and proliferation by activating EphA2 receptor phosphorylation at Ser897 and downstream PIK3R2/GSK3b/β-catenin positive feedback loop in HCC cells.

EphrinA5

EphrinA5, as the ligand of EphA4, also exhibited a tumor-suppressive effect as expect. Wang et al. identified two alternative isoforms of ephrinA5 in clinical samples, a large isoform (ephrinA5L) and a small isoform (ephrinA5S) [66]. Both ephrinA5S and ephrinA5L were down-regulated in HCC samples, but only expression of ephrinA5S was significantly correlated with prognosis of patients after resection. Moreover, ephrinA5S showed a more pronounced inhibitory effect compared with ephrinA5L on cellular proliferation and migration *in vitro*. The anti-tumor effect of ephrinA5 was attributable to c-Cbl-mediated EGFR degradation in HCC [66].

EphrinBs

The mRNA level of the ephrinB1 was significantly higher in HCC samples than in normal liver, while the differences in expression of ephrinB2 and ephrinB3 were not obvious [67]. *In vivo*, the up-regulation of ephrinB1 accelerated tumor growth and increased tumor vessel number. In addition, in vitro experiments confirmed that ephrinB1 promoted neovascularization by inducing proliferation and migration of human umbilical vein endothelial cells (HUVECs) [67].

4 Therapeutic Potential of Eph/Ephrin-Targeted Drugs in HCC

Over the last decade, advances in systemic therapy have contributed to tremendous improvements for patients diagnosed with advanced-stage HCC or patients who experienced failures in other therapies. As our knowledge of the molecular profiling changes regulating tumorigenesis evolves, some targeted drugs have been developed and been implemented in the clinic, which ushered a new era in treatment in HCC. A few drugs have been confirmed to significantly improve prognosis of patients with HCC compared with placebo, such as sorafenib and regorafenib [68–70]. However, drug resistance is frequently observed especially in patients who receiving long-term corresponding drugs treatment [71,72]. Therefore, the identification of novel targets and prognostic predictors in HCC remains crucial and will facilitate the shift towards individualized precision medicine.

As described above, multiple members of Eph/Ephrin system are involved in oncogenesis and associated with clinical outcome of patients in HCC. Blocking tumor-promoting Eph/Ephrin-related signal pathways could offer an effective strategy for treatment in HCC. Many Eph/Ephrin system-targeted drugs have been developed and investigated in cancers, such as antibodies, recombinant proteins and small molecules [18,73]. Some of these drugs have entered to clinical trials (Table 1). However, among them only sEphB4-HSA was evaluated in HCC in a phase I study, which reported that 1 of 8 HCC patients had a partial response [74].

Table 1: Eph/Ephrin system-targeted drugs for cancer therapy in clinical trials

Drug	Target	Category	Cancers	Reference/ NCT number
sEphB4-HSA	EphrinB2	Recombinant albumin fusion protein	Solid and hematologic malignancies	[74]
DS-8895a	EphA2	Antibody	Solid tumors	[75,76]
MEDI-547	EphA2	Antibody-drug conjugate	Solid tumors	[77]
MM310	EphA2	Antibody-drug conjugate	Solid tumors	[78]
BT5528	EphA2	Bicycle toxin conjugate	Solid tumors	[79]
EphA2-targeting DOPC-encapsulated siRNA	EphA2	siRNA	Solid tumors	NCT01591356
KB004	EphA3	Antibody	Hematologic malignancies	[80]
PF-06647263	ephrinA4	Antibody-drug conjugate	Solid tumors	[81]

On the other hand, results from preclinical studies provided novel promising strategies for design and selection of Eph/Ephrin system-targeted drugs in HCC treatment (Table 2). Yamaguchi et al. reported that EphA2-derived peptide vaccine with amphiphilic poly nanoparticles (Eph-NPs) exhibited an anti-tumor effect against liver cancer in mice [82]. Immunization with Eph-NPs promoted generation of EphA2-specific CD8+ T cells without any toxic damage to liver [82]. As a selective EphA2 small molecular inhibitor, ALW-II-41–27 was recently confirmed to have anti-tumor effects in many malignant tumors [83–85]. In HCC, ALW-II-41–27 significantly impaired the capacity of cellular proliferation *in vitro* [38]. Due to the specific tropism of rAAV-8 for liver *in vivo*, rAAV-8-shEphB2 was established by et al. for treatment of HCC [58,86]. In immunocompetent mice, rAAV-8-shEphB2 increased sensitivity of HCC to sorafenib [58].

Table 2: Eph/Ephrin system-targeted drugs for HCC therapy

Drug	Category	Target	Effect	Reference
Eph-NPs	Vaccine	EphA2	Promotes generation of EphA2-specific CD8+ T cells	[82]
ALW-II-41–27	Small molecule	EphA2	Impairs the capacity of cellular proliferation *in vitro*	[38]
rAAV-8-shEphB2	AAV	EphB2	Increases sensitivity of HCC to sorafenib	[58]

5 Conclusion and Perspective

Accumulative evidence demonstrated that Eph receptors and their corresponding ephrin ligands had an important role in the initiation and development of HCC. In general, Ephs and ephrins exhibited a pro-tumorigenic effect in HCC by influencing proliferation, apoptosis, migration, invasion, angiogenesis, stemness and drug/radiotherapy resistance, although there are exceptions (EphA4 and ephrinA5 as tumor suppressors). However, the precise mechanisms and roles of Eph/Ephrin in HCC remain unclear. Specifically, the reverse signaling of ephrins is almost never mentioned in HCC. Besides, given the key function of the system in immunity, the association between Eph/Ephrin and tumor immune microenvironment in HCC deserves further exploration in order to offer possible strategies for immunotherapy. Furthermore, although their therapeutic potential is promising, the high promiscuity between Ephs and ephrins can easily surmount the selective blockage of a single target. Thus, designing multitargeted anti-Eph/Ephrin drugs may be a better strategy. For example, UniPR1331 has been identified as a broad-spectrum antagonist of the Eph/Ephrin system, and its effect on HCC should be further investigated in the future. On the other hand, due to the ubiquitous expression of the system in various tissues, non-specific targeting should be avoided.

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