Review

Emerging and Diverse Functions of the EphA2 Noncanonical Pathway in Cancer Progression

Yue Zhou* and Hiroki Sakurai*
*Department of Cancer Cell Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; Toyama 930–0194, Japan; and The MOE Key Laboratory for Standardization of Chinese Medicines and the Shanghai Key Laboratory of Compound Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine; Shanghai 201203, China.

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Erythropoietin-producing hepatocellular receptor A2 (EphA2) receptor tyrosine kinase controls multiple physiological processes to maintain homeostasis in normal cells. In many types of solid tumors, it has been reported that EphA2 is overexpressed and plays a critical role in oncogenic signaling. However, in recent years, the opposing functions of EphA2 have been explained by the canonical and noncanonical signaling pathways. Ligand- and tyrosine kinase-dependent EphA2 activation (the canonical pathway) inhibits cancer cell proliferation and motility. In contrast, ligand- and tyrosine kinase-independent EphA2 signaling (the noncanonical pathway) promotes tumor survival and metastasis and controls acquired drug resistance and maintenance of cancer stem cell-like properties. Evidence has accumulated showing that the EphA2 noncanonical pathway is mainly regulated by inflammatory cytokines and growth factors via phosphorylation at Ser-897 in the intracellular C-tail region via some serine/threonine kinases, including p90 ribosomal S6 kinase. In this review, we focus on the regulation of Ser-897 phosphorylation and its functional importance in tumor malignancy and discuss future therapeutic targeting.

Key words receptor tyrosine kinase; noncanonical pathway; tumor malignancy; erythropoietin-producing hepatocellular receptor A2 (EphA2)

1. INTRODUCTION

The ephrin (Eph) receptors are the largest family of receptor tyrosine kinases (RTKs) and they mainly regulate cell proliferation and migration during development as well as tissue homeostasis, for example, axon guidance, synapse plasticity, tissue remodeling, bone morphogenesis, and angiogenesis.1-6 The first Eph receptor was cloned from an erythropoietin-producing hepatocellular cancer cell line in 1987, which was named EphA1, and subsequently erythropoietin-producing hepatocellular receptor A2 (EphA2), the most widely characterized member, was identified in 1990 by the screening of the cDNA library of HeLa cells.7,8 While it is expressed in normal epithelial cells, including the skin, kidney, liver, lung, small intestine, colon, and lens, the expression of EphA2 is inhibited in these differentiated tissues.9,10 On the other hand, various solid tumors, such as breast, ovary, prostate, pancreas, glioblastoma, neck, renal, lung, melanoma, bladder, gastric esophageal, colorectal, and cervical cancers, were reported to express high levels of EphA2.11-17 Of note, its expression is associated with a more aggressive cancer phenotype and correlated with tumor metastasis and poor patient survival.6,12,13,17-19 Recent reports have provided evidence that EphA2 is involved in the promotion of the epithelial-mesenchymal transition (EMT) and maintenance of cancer stem cell-like properties.20-22 For example, EphA2-deficient mice or EphA2-knockdown cancer cells display impaired tumor development, metastasis, and angiogenesis.23-26 Moreover, EphA2 has been reported to engage in cross-talk with other RTK family members to promote tumor malignancy.27-32 For example, it is involved in the resistance to ErbB tyrosine kinase inhibitor (TKI) or monoclonal antibodies, and EphA2 inhibition could become a new strategy to restore anti-ErbB sensitivity. Hence, many reports identified EphA2 as a critical tumorigenic and tumor-promoting factor.

However, in contrast, some studies found that EphA2 reduces cancer cell proliferation and motility, suggesting that EphA2 has both pro- and anti-oncogenic functions.19,33 In 2009, Miao et al. found that EphA2 tyrosine kinase activity, which is induced by EphA2 ligands, inhibits the migration of cancer cells, whereas Akt phosphorylates EphA2 on Ser-897 in an EphA2 tyrosine kinase activity-independent manner, and this novel Akt–EphA2 signaling induces human glioblastoma malignancy.34 Interestingly, EphA2 ligands, including ephrin-A1, were reported to be attenuated in many types of aggressive tumor cells, especially in those expressing high levels of EphA2. This inhibition appears to be controlled by extracellular signal-regulated kinase (ERK) activation. Taken together, the EphA2 tyrosine kinase-dependent signal (canonical) pathway plays mainly antioncogenic roles, and the tyrosine kinase-independent signaling (noncanonical) pathway should be essential for tumor malignant alteration. This review mainly focuses on the emerging and diverse roles of the noncanonical EphA2 pathway.

2. BASIC STRUCTURE OF EphA2 AND EphA2 CANONICAL PATHWAY

Eph family receptors are single transmembrane proteins divided into two classes, EphA and EphB, depending on the homology of their extracellular domains.1-6,9,15,17-19 Nine EphA receptors and five EphB receptors sharing a common structure

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are encoded in the human genome (Fig. 1). A ligand-binding domain, Sushi domain, epidermal growth factor (EGF)-like domain, two fibronectin type-III repeats, juxtamembrane region, tyrosine kinase domain, sterile alpha motif (SAM) and PDZ domain-binding motif. There are eight ligands of the Eph receptor, called ephrin, which is also divided into the A and B subclasses. Five types of ephrinA ligands are anchored to the cell membrane by a glycosylphosphatidylinositol linkage, while three types of ephrinB ligands are located on the cell membrane via a transmembrane helix.

The interaction between ephrin and the Eph receptor results in both “forward signaling” and “reverse signaling,” which mediate the Eph receptor and ephrin, respectively.1–6,9–15,17–19 Forward signaling induces Eph receptor oligomer clustering, cross-phosphorylation of each other’s tyrosine residues on the juxtamembrane domain and activation loop, and, consequently, evokes kinase activity. Some proteins, including a Ras family guanosine triphosphatase (GTPase)-activating protein, guanine-nucleotide exchange factors, Vav, ephexin, focal adhesion kinase (FAK), Src family cytoplasmic tyrosine kinases, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K), have been reported to interact with activated receptors and regulate the signaling. On the other hand, ephrin mediates reverse signaling through the association with some transmembrane proteins, such as p75NTR, TrkB, and Ret receptor tyrosine kinases.

In the case of EphA2, the most preferred ligand is ephrin-A1.1–6,9–15,17–19 Normally, EphA2 binds to ephrin-A1 on its neighboring cells and influences cell proliferation, survival, migration, morphology, cell–cell repulsion and adhesion in

**Fig. 1. Structure and Function of the EphA2 Canonical Pathway**

EphA2 consists of the ligand-binding domain, Sushi domain, epidermal growth factor (EGF)-like domain, two fibronectin type-III transmembrane domains, juxtamembrane region, tyrosine kinase domain, SAM, PSD-95 postsynaptic density protein, Discs large, and PDZ domain-binding motif. Its ligand ephrin-A1 is anchored to the cell membrane by a glycosylphosphatidylinositol linkage. After binding to ephrin-A1, EphA2 forms oligomers and induces EphA2 forward signaling and ephrin-A1 reverse signaling. These signals control many types of physiological processes.

**Biography**

Dr. Yue Zhou is associate professor at the MOE Key Laboratory for Standardization of Chinese Medicines and the Shanghai Key Laboratory of Compound Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine. She is also a research fellow at Department of Cancer Cell Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama. She received her Ph.D. in Pharmaceutical Sciences at University of Toyama in 2014, and then, continued her research at University of Toyama as a postdoctoral researcher. In 2015, she moved to Department of Bioregulation and Cellular Response, Graduate School of Medicine, Osaka University as a postdoctoral researcher, and took up her present position in 2016. Her main research interest is the molecular mechanisms of tumour progression via inflammatory signalling pathways. Currently, she tries to find a novel crosstalk between the inflammatory signalling pathway and the non-canonical signalling pathway of receptor tyrosine kinases, and tries to discover natural products which are able to inhibit the two signalling pathways.
embryonic development, axon guidance, synaptogenesis, angiogenesis, and casculogenesis (Fig. 1). Wiedemann et al. reported that ephrin-A1 expression is associated with cell density in endothelial cells. In the confluent state, ephrin-A1 is highly expressed and binds to EphA2, inducing its activation to inhibit cell proliferation, growth arrest, and migration in vascular endothelial cells. In contrast, ephrin-A1 is inhibited to promote cell proliferation and migration in low cell density, suggesting the existence of ephrin-A1-independent EphA2 signaling.

Ephrin-A1-induced EphA2 activation was shown to inhibit FAK, Akt, and ERK phosphorylation to control cell motility, viability, and proliferation in various cancer cell lines. Of note, in some types of cells, the canonical pathway was reported to promote ERK activation via SH2-containing collagen-related proteins (SHC) and growth factor receptor-bound protein 2 (GRB2). This disparity highlights how the cell type and cell microenvironment affect signaling.

Recently, Neill et al. have reported a novel EphA2 ligand, progranulin. Progranulin is an evolutionarily conserved cystein-rich secreted glycoprotein and expressed ubiquitously. Differing from ephrin-A1, progranulin induces ERK and Akt phosphorylation to regulate the capillary morphogenesis of human umbilical vein endothelial cells (HUVECs). Additionally, they found a positive feedback loop of progranulin expression dependent on the progranulin/EphA2 signaling pathway. Most reports showed that ephrin-A1 exhibits antioncogenic properties, although progranulin expression is positively related to tumorigenesis.

EphA2 was suggested to be a direct transcriptional target of the Ras–Raf–ERK pathway. On the other hand, ephrin-A1/EphA2 interaction can attenuate the growth factor-induced activation of Ras. Thus, there is a negative feedback loop to regulate Ras activity. Dunne et al. reported that EphA2 is overexpressed in colorectal cell lines with the KRAS or BRAF active mutation. Yeddula et al. determined that EphA2 acts with KRAS to suppress tumor proliferation by inhibiting Akt and ERK phosphorylation as well as Hedgehog signaling, and the proliferation of lung adenocarcinoma was promoted with the loss of EphA2 expression in KRASG12D mice. Therefore, the Ras–Raf–ERK pathway and ephrin-A1–EphA2 pathway involve crosstalk to regulate tumor malignancy. Hence, EphA2 canonical signaling mainly plays a role as a tumor suppressor, although in some cases it also has a tumor-initiating function.

3. NONCANONICAL PATHWAY OF EphA2

3.1. EphA2 Noncanonical Signaling Transduction
EphA2 canonical signaling depends on ephrin-A1 binding and its tyrosine kinase activity to preserve the status of normal epithelial cells. However, ephrin-A1 is often inhibited in many types of tumor tissue. The Ras–Raf–ERK pathway inhibits the expression of ephrin-A1, whereas it promotes EphA2 expression. Many reports showed that patients with low ephrin-A1 expression had a poor prognosis, suggesting the existence of EphA2 tyrosine kinase-independent regulation. Thirty-seven phosphorylation sites are identified within the intracellular domain of EphA2 in the PhosphoSitePlus database. Among them, 25 phosphorylation sites are serine and threonine residues. EphA2 Ser-897, which is located in the linker region between the kinase domain and SAM domain, plays critical functions in the noncanonical signaling pathway (Fig. 1). In 2009, the first report of the molecular regulation of Ser-897 phosphorylation was published. Miao et al. used anti-phospho-Akt substrate antibody or anti-phospho-EphA2 (Ser-897) antibody and found that Akt phosphorylates EphA2 on Ser-897. Akt activation promoted by serum stimulation or multiple growth factors strongly induced EphA2 Ser-897 phosphorylation in glioma cell lines. In addition, immunofluorescent staining images showed that EphA2 and phospho-Akt were co-localized in glioma cell lines. Moreover, EphA2 phosphorylation on Ser-897 and Akt phosphorylation on Ser-473 overlapped in human glioma specimens. Taken together, those results indicate that EphA2 Ser-897 phosphorylation is catalyzed by Akt.

In 2015, we demonstrated that p90 ribosomal S6 kinases...
(RSK), mainly RSK1 and RSK2, are major kinases of EphA2 catalyzing Ser-897 phosphorylation.\(^{45}\) RSK is a member of the AGC family kinases, and Akt also belongs to the same family. Both RSK and Akt share substrate specificity characterized by arginine (Arg) at position −3 relative to the phosphorylated serine/threonine. Not only serum stimulation or growth factors but also inflammatory cytokines induce EphA2 Ser-897 phosphorylation, and this phosphorylation is completely inhibited by the RSK inhibitor, but not by PI3K/Akt inhibitors, in cervical cancer, glioma, breast cancer, lung cancer, and colorectal cancer cells. In addition, the results of in vitro kinase assays, RSK-knockdown experiments, and RSK/EphA2-overexpression experiments indicated that RSK is the major kinase for EphA2 Ser-897 phosphorylation. Moreover, EphA2 Ser-897 phosphorylation and RSK Ser-380 phosphorylation overlapped in cultured cell lines as well as multitudinous tissue specimens. A recent report has shown that EphA2 Ser-897 can be phosphorylated by protein kinase A (PKA), but not by Akt, in forskolin-stimulated prostate cancer cells.\(^{46}\) PKA also belongs to the AGC family and has a similar substrate recognition motif, suggesting that other AGC protein kinases may catalyze EphA2 Ser-897. It was also shown that not all prostate and pancreatic cancer cell lines, which those authors used in their experiments, \(^{46}\) were sensitive to forskolin. However, EGF-induced phosphorylation of EphA2 on Ser-897 was detected in all cell lines, suggesting that the cellular context and cell environment influence the kinase of EphA2 Ser-897. Overall, EphA2 Ser-897 phosphorylation is controlled by AGC protein kinases and, depending on the cellular context and type of stimulation, RSK, Akt, PKA, and other AGC family members appear to be selected.

In 2013 and 2015, two groups reported that EphA2 is cleaved by a membrane-bound matrix metalloprotease, MT1-MMP.\(^{47,48}\) Although Sugiyama et al.\(^{47}\) showed that the cleavage site was Y\(^{385}\)-I and T\(^{395}\)-I and Koshikawa et al.\(^{48}\) found that it was S\(^{-427}\)-F and S\(^{-432}\)-V, cleaved EphA2 had a ligand-insensitive form to promote cancer migration. Therefore, EphA2 cooperates with MT1-MMP to play a role as a tumor initiator.

Interestingly, not only Ser-897 but also Ser-901 is phosphorylated by PKA upon forskolin stimulation.\(^{46}\) Ser-901 is also located in the same linker region, and there are three other serine/threonine phosphorylation sites. This region should play a main role in EphA2 noncanonical activation, and therefore a detailed study of the five phosphorylation sites will be important to understand EphA2 noncanonical regulation fully. Additionally, the SAM domain was reported to be required for Ser-897 phosphorylation and its noncanonical function, suggesting that the SAM domain stabilizes the kinase approach or inhibits the phosphatase approach to Ser-897.\(^{49,50}\) Moreover, EphA2 prefers the dimer formation in the absence of ephrin-A1. This dimerization suppresses the phosphorylation of Ser-897 and attenuates the function of Ser-897 in comparison with the EphA2 monomer, indicating that the substrate-kinase reaction for EphA2 on Ser-897 is more stable in the EphA2 monomer than in the dimer.

Meanwhile, one report showed that ephrin-B3 regulates the Akt–EphA2 signaling pathway.\(^{51}\) EphA2 expression and phosphorylation on Ser-897 were inhibited in the ephrin-B3 knockdown U-1810 non-small cell lung carcinoma (NSCLC) cell line. Interestingly, Akt phosphorylation was controlled by ephrin-B3 as well. Hence, there are still many structural and functional mysteries related to the EphA2 serine/threonine phosphorylation residues located in the linker region between the kinase and SAM domains, and detailed research will be required to understand the regulation of the EphA2 noncanonical signaling pathway.

### 3.2. Cell Motility and Cell Morphology

The best-known function of the noncanonical signaling of EphA2 is controlling cell motility and cell morphology (Fig. 3). Miao et al. demonstrated that EphA2 Ser-897 regulates growth factor-induced chemotactic glioma cell migration and invasion.\(^{34}\) EphA2 Ser-897 mainly localizes in the migration front with dendritic actin in lamellipodia or the tips of F-actin fibers, but not in cell–cell contact junctions, suggesting that EphA2 Ser-897 phosphorylation promotes the assembly of the actin cytoskeleton and extension of lamellipodia. We demonstrated that the RSK–EphA2 axis also induces cell motility.\(^{45}\) The RSK inhibitor or RSK knockdown suppressed the migration and invasion of breast cancer cells. Similar results were obtained using siRNAs against EphA2. In addition, cell migration reduced by EphA2 knockdown was recovered by re-expression of kinase-dead EphA2, but not by its Ser-897- Ala mutant, indicating that RSK-mediated Ser-897 phosphorylation of EphA2 is indispensable for cell motility. Moreover, we also found that EphA2 Ser-897 and total EphA2 were localized in the migrating front with F-actin in lamellipodia. Interestingly, the RSK inhibitor not only inhibited staining of EphA2 Ser-897 but also collapsed its elongated and polarized morphology, suggesting that RSK controls cell motility by maintaining pS-EphA2 localization at the edge in the direction of movement, such as in lamellipodia. Taking all the results together, it appears that AGC family-induced EphA2 Ser-897 phosphorylation promotes cell migration and invasion.

Recently, Gundry et al. have demonstrated that EphA2 Ser-897 phosphorylation is necessary for EphA2 trafficking through the RCP/Rab14 pathway to maintain cell–cell repulsion.\(^{52}\) They showed that EphA2 was constitutively internal-
ized and returned to the cell membrane rapidly. However, upon hepatocyte growth factor (HGF) stimulation, the Akt–EphA2 pathway as well as the LMTK3–RCP pathway was activated. LMTK3 is a transmembrane serine/threonine kinase, and RCP is a Rab effector. LMTK3 is activated by HGF and induces RCP Ser-435 phosphorylation. Then, phosphorylated RCP interacts with Rab14. This complex is associated with the endosome and required for slower EphA2 recycling to the cell membrane, but not for normal rapid recycling. Rapid EphA2 internalization and recycling does not initiate EphA2 functional signaling transduction but only maintains the storage of EphA2 on the cell surface, and this slower recycling would promote cytoskeletal responses, cell–cell repulsion, and cell scattering. Both the LMTK3–RCP signaling pathway and Akt–EphA2 signaling pathway were necessary for efficient invasion and metastasis in an in vivo model of pancreatic cancer.52)

It is well known that the Rho family of small GTPases plays critical roles in the regulation of cell motility and morphology.53–55) Kawai et al.56 demonstrated that the interaction of EphA2 and Ephexin4, which is one of the RhoG guanine nucleotide exchanges, was induced in an EphA2 Ser-897 phosphorilation-dependent manner.56) Ser-897 phosphorylation was pivotal for RhoG activity, and activated RhoG bound to its effector ELMO and promoted the activation of Rac to promote cell motility. RhoG also bound to PI3K and regulated the PI3K–Akt pathway, which is associated with tumor malignancy and the EMT. The same group also found that EphA2/EphB6 interaction inhibited EphA2 noncanonical signaling.57) EphB6 is frequently silenced in metastatic tumor tissues and was reported to be a tumor suppressor. Kawai et al.56) proposed that the heterodimer formation of EphA2 and EphB6 inhibited EphA2 Ser-897 phosphorylation, followed by the suppression of the EphA2/Ephexin4 interaction, promoting anoikis.

Meanwhile, Cui et al. identified the Akt–mammalian target of rapamycin complex 1 (mTORC1), Raf–MEK–ERK, and Pyk2–Src–ERK pathways as the downstream signaling of the EphA2 noncanonical pathway in cholangiocarcinoma cells.58) They used the kinase inhibitor to determine their function, and found that the Src inhibitor inhibited cell migration in EphA2-overexpressing cells, suggesting the Src–ERK pathway mainly controls cell migration. In addition, cleaved EphA2 processed by MT1-MMP also activates the Ras–ERK and PI3K–Akt pathways, even when ephrin-A1 is overexpressed.57,59) Notably, Koshikawa et al. reported that phospho-Ser-897 EphA2 and MT1-MMP were co-localized in ovarian carcinoma, but not in the normal ovary.49) In addition, cleaved EphA2 was required for tumor growth and metastasis of A431 cells in vivo.50) Moreover, in human invasive cutaneous squamous cell carcinoma tissue, EphA2 was also reported to be processed by MT1-MMP, and cleaved EphA2 was positively related to lymph node metastasis.59) Therefore, the noncanonical pathway of EphA2 appears to play pivotal functions in MT1-MMP-expressing human tumor microenvironments.

Barquilla et al.50) determined that PKA–EphA2 signaling inhibits cell retraction, a prototypical repulsive response. Retraction was induced by ephrin-A1 stimulation in a prostate cancer cell line, and forskolin or PKA agonist inhibited cell retraction via EphA2 phosphorylation at Ser-892, -897, and -901, suggesting that other phosphorylation sites located in the linker region also contribute to the EphA2 noncanonical pathway.50)

Not only in cancer cells but also in normal cells, cell motility depends on the noncanonical signaling pathway. Wiedemann et al. reported that ephrin-A1 expression was decreased in low-density HUVEC cultures in which EphA2 Ser-897 instead of Tyr-588 was phosphorylated to regulate cell motility and proliferation.56) They propounded a model in which the expression gradient of ephrin-A1, which is promoted by lower cell density in injured areas, determines the direction of cell migration. The leading cells at the wound barrier show lower ephrin-A1 expression, combined with less EphA2-phosphorylation of Tyr-588 and greater EphA2-phosphorylation of Ser-897, and develop migratory activity toward the injured zone.

Harada et al. investigated the noncanonical signaling pathway in the formation of epithelial structures using three-dimensional Madin–Darby canine kidney (MDCK) cell cultures.60) HGF stimulation induced MDCK cysts via EphA2 Ser-897 phosphorylation. In this process, phospho-Ser-897 EphA2/Ephexin4/RhoG activation was required. Their results suggested EphA2 Ser-897 phosphorylation plays a pivotal role in the formation and maintenance of epithelial tissues. Taken together, EphA2 Ser-897 phosphorylation is also necessary in physiological reactions.

In addition to Ser-897 phosphorylation, a recent report has shown that Tyr-772 phosphorylation, which is located in the activation loop, has both EphA2-tyrosine kinase activity-dependent and -independent functions.61) Locard-Paulet et al. used a phosphoproteomic strategy to detect the bi-directional signaling pathways between human metastatic breast cancer cells and endothelial cells and found EphA2 Tyr-772 phosphorylation was decreased in cancer cells upon endothelial cell contact and it was critical for EphA2-mediated inhibition of transendothelial migration and cancer cell adhesion. Moreover, Tyr-772 was phosphorylated during the early phase of ephrin-A1 stimulation, but its dephosphorylation was observed in the late phase only in breast cancer cells with a high rate of metastasis to the lung, but not in the parental breast cancer cells. Their model for Tyr-772 regulation is as follows: in the early phase, ephrin-A1 on endothelial cells phosphorylates EphA2-Tyr-772 on cancer cells to induce cell–cell repulsion, thereby inhibiting tumor cell–endothelial cell adhesion and transendothelial cell migration. In the late phase, Tyr-772 phosphorylation is reduced by protein tyrosine phosphatase low molecular weight protein tyrosine phosphatase (LMW-PTP) and revokes ligand-driven EphA2 signaling to promote transendothelial migration and cancer cell adhesion.

3.3. Cell Survival and Proliferation Hamaoka et al. investigated the RSK–EphA2 signaling pathway in the proliferation of glioblastoma cells.62) Glioblastoma cells often overexpress EphA2, which is associated with tumor malignancy and poor patient prognosis. The proliferation of EGF-stimulated U-251 and A172 cells was suppressed by EphA2 knockdown or ephrin-A1 stimulation, which promotes EphA2 degradation, indicating that EphA2 is required for cell proliferation. In these cells, RSK, but not Akt, controlled EphA2 Ser-897 phosphorylation. Similar to EphA2 knockdown, RSK inhibitor or RSK knockdown attenuated cell proliferation. The cells also overexpressed wild-type EphA2 or the Ser-897-Ala EphA2 mutant with RSK2 in HEK293 cells, and cell proliferation was
promoted by the wild type, but not by the Ser-897-Ala-mutant. Taking all the results together, the phosphorylation of EphA2 on Ser-897 via RSK promotes glioblastoma cell proliferation.

As described above, the Akt–mTORC1, Raf–MEK–ERK, and Pyk2–Src–ERK signaling pathways were identified as the downstream signaling of the EphA2 noncanonical pathway. Although the effects of c-Src on cell proliferation were weaker than those of the other factors, the authors concluded that all three signaling pathways promoted cell proliferation in an EphA2-dependent manner.

3.4. Tumor Malignancy
High EphA2 expression positively correlates with tumor stage, progression, and patient survival. Miao et al. found that EphA2 Ser-897 phosphorylation was expressed mainly in grade IV human glioma specimens, suggesting its correlation with glioma malignancy, and the staining was strongly present in the region of growth factor enrichment and invasive cells. In addition, Akt Ser-473 phosphorylation was stained in the adjacent section, indicating that Akt induced EphA2 Ser-897 phosphorylation in human specimens. Of note, EphA2 Ser-897 phosphorylation was detected in a tumor that had infiltrated the meninges adjacent to normal brain tissue. This result supports EphA2 promotion of tumor cell migration.

We investigated RSK Ser-380 phosphorylation and EphA2 Ser-897 phosphorylation using immunohistochemical methods in a multiple cancer tissue microarray, which included 13 organ cancer tissues, and found double-positive samples in colorectal, stomach, ovary, liver, uterine corpus, and lung cancer specimens, indicating that EphA2 Ser-897 phosphorylation and RSK Ser-380 phosphorylation co-localized in tumor tissues. The overlapping expression of these two phosphorylation factors was also observed in lung adenocarcinoma tissues with activated EGFR mutations, including exon 19 deletion, in which EphA2 Ser-897 phosphorylation was stained mainly in the plasma membrane. We also explored the role of the RSK–EphA2 pathway in patient prognosis using a lung cancer tissue microarray. There were no significant differences between clinicopathological factors, including smoking, and the expression of the RSK–EphA2 pathway; however, EphA2/RSK double phosphorylation-positive patients had shorter overall survival. Moreover, a drastic difference was observed in smoking patients. Taken together, the reports strongly suggest that the EphA2 noncanonical pathway promotes tumor malignancy, and inhibition of EphA2 phosphorylation on Ser-897 will be a novel therapeutic target in various tumors.

3.5. Cancer Stemness
EphA2 was reported to be overexpressed in human glioblastoma cancer stem cells (CSCs) and required to maintain self-renewal and an undifferentiated state.

Its expression correlated with the size and tumor-initiating ability of CSCs, and EphA2 depletion by either ephrin-A1 treatment or EphA2 silencing attenuated self-renewal and tumorigenicity. In melanoma cells, EphA2 is also involved in cancer cell stemness. Melanoma cells expressing stemness markers formed melanospheres, and they had self-renewal and tumor-initiating ability. Additionally, EphA2 expression is positively correlated with aldehyde dehydrogenase (ALDH), which is one of the CSC markers, in lung cancer. Dunne et al. found that EphA2 Ser-897 and EphA2 Tyr-772 as well as total EphA2 were upregulated in an invasive colorectal cancer cell line harboring the KRAS active mutation. Of note, the stem cell markers CD44 and Lgr5 also were increased in these cells, suggesting EphA2 Ser-897 may be involved in cancer stemness. De Robertis et al. reported that colon adenocarcinoma cells with high EphA2 expression levels isolated from the AOM/DSS mouse model showed higher levels of stem cell markers and lower levels of differentiation markers than the low-EphA2 population. At the same time, ephrin-A1 was decreased in the high-EphA2 population, suggesting that the noncanonical pathway is responsible for cancer stemness properties. Miao et al. reported that the Akt–EphA2 pathway is essential for glioblastoma CSC invasion and self-renewal.

They found that glioblastoma CSCs overexpressing EphA2-WT were much more invasive than EphA2-Ser-897-Ala mutant cells in vivo. In addition, the stemness marker Sox2 was attenuated in EphA2-Ser-897-Ala mutant cells, and glioblastoma CSC neurosphere-formation ability was also inhibited in vitro. Collectively, the results suggest that the EphA2 noncanonical signaling plays a critical role in maintaining cancer stemness properties.

3.6. Resistance to Molecular-Targeted Agents
EphA2 has been reported to be involved in acquired resistance to EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib, afatinib, or EGFR monoclonal antibodies. For example, Koch et al. demonstrated that EphA2 was overexpressed in gefitinib-resistant NSCLC, and the multikinase inhibitor dasatinib overcame the resistance, suggesting the involvement of the EphA2 noncanonical pathway via Akt or RSK. Patients with colorectal cancer with wild-type KRAS are often treated with the EGFR monoclonal antibody cetuximab. It was reported that high EphA2 receptor expression was associated with a worse outcome in patients treated with cetuximab. De Robertis et al. found that EphA2 and EGFR overexpression showed a combination effect relative to cetuximab resistance, independent of the KRAS mutation status.

Amato et al. compared EGFR TKI-sensitive with EGFR TKI-resistant EGFR-mutant lung cancer cell lines and determined that EphA2 Ser-897 and Tyr-588 phosphorylation as well as total EphA2 expression were markedly increased in the resistant cells. Notably, EphA2 levels were higher in post-relapse tumor sections than in TKI-pretreatment tumor sections from four patients with the EGFR mutation. As expected, EphA2 knockdown reduced the cell viability of resistant cells in vitro. In addition, EphA2 silencing decreased the tumor burden and increased the survival time in TKI-resistant EGFR<sup>KRAS<sup>WT</sup> or KRAS<sup>790M</sup></sup> transgenic mice, which were dependent on decreasing proliferation and increasing apoptosis in the resistant tumors, respectively. EphA2 silencing increased caspase 3 and poly(ADP-ribose) polymerase (PARP) cleavage to induce cell apoptosis in vitro, and decrease RSK, S6 kinase 1 (S6K1), and BAD phosphorylation to inhibit tumor cell proliferation. Amato et al. previously screened the compounds predicted to inhibit EphA2 tyrosine kinase activity and identified ALW-II-41-27 as an EphA2 TKI. ALW-II-41-27 is a type II small-molecule inhibitor targeting the ATP-binding pocket of the kinase domain and an allosteric site next to the DFG motif in the receptor. This compound inhibited EphA2 Tyr-588, as well as Akt, RSK, S6K1, S6, and BAD phosphorylation, to inhibit cancer viability in vitro in NSCLC cell lines, and inhibited NSCLC tumor growth by promoting tumor cell apoptosis and suppressing cell proliferation in vivo. In the EGFR TKI-resistant cells, ALW-II-41-27 suppressed cell viability and...
proliferation and promoted apoptosis. In addition, it decreased the tumor growth of resistant cells in vivo. Remarkably, ALW-II-41-27 inhibited EphA2 phosphorylation on Ser-897 via RSK or Akt inhibition both in vitro and in vivo, suggesting that EphA2 Ser-897 may be essential for cell viability in EGFR TKI-resistant NSCLC tumor cells. Collectively, EphA2 Ser-897 phosphorylation serves as a useful therapeutic target in TKI-resistant tumors.

4. CONCLUSION

EphA2 has been reported to be both a tumor initiator and suppressor. As shown in this review, the tumor-initiator function is mainly dependent on the EphA2 noncanonical pathway via AGC kinase-mediated Ser-897 phosphorylation and it controls cancer motility, proliferation, stemness properties, and drug resistance to promote tumor malignant progression. This noncanonical pathway will be a novel strategic approach for targeted cancer therapies and tumor diagnostics. While the canonical pathway has been considered to be a form of tumor-suppressive signaling, some recent reports have identified its critical role in single-cell invasion and the shift to the mesenchymal-to-amoeboïd transition. Additionally, the EphA2 G391R mutation has been detected in lung squamous cell carcinoma. This mutation not only induces EphA2 tyrosine activation but also promotes cell invasion, focal adhesion, and cell survival. Is the EphA2 noncanonical pathway involved in these phenomena? Further studies are needed to understand fully the diverse functions of EphA2 in cancer progression.

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Conflict of Interest The authors declare no conflict of interest.

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