Axl Receptor Axis: A New Therapeutic Target in Lung Cancer

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Axl belongs to the TAM family of receptor tyrosine kinases, which consists of Tyro3, Axl, and Mer. All three family members have similar structures and share a number of ligands, including the vitamin K–dependent ligands growth arrest protein 6 (Gas6) and protein S. In normal tissues, TAM receptor tyrosine kinases contribute to immune response regulation, including clearance of apoptotic cells and inhibition of cytotoxic immune activation in response to apoptosis. When cells undergo apoptosis, the polarity of the plasma membrane lipid bilayer is altered, externalizing the anionic phospholipid phosphatidylserine (PS). Gas6, which is often prebound to Axl, binds PS via the γ-carboxyglutamic domain. This ligand-dependent Axl activation regulates macrophage-mediated endocytosis and clearance of apoptotic cells by a process termed *efferocytosis* while inhibiting proinflammatory cytokine response.[1] In preclinical models, TAM receptor triple-knockout mice (Tyro3-/-, Mer-/-, and Axl-/-) develop normally, but as the immune system matures, chronic inflammation and autoimmunity tends to develop. TAM receptor tyrosine kinases also participate in platelet activation and clot stability.[2] Other less-studied mechanisms of Axl activation include ligand-independent homodimerization of Axl due to receptor overexpression, transcellular homophilic binding of the Axl extracellular domain, heterodimerization with other TAM family receptors such as Tyro3, and dimerization with non-TAM receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) (Fig. 1).[3–6]

Complex transcriptional and translational mechanisms regulate Axl expression (see Fig. 1). The AXL receptor tyrosine kinase gene (*Axl*) is located on chromosome 19 and consists of 20 exons. Different Axl transcripts arise from alternative splicing of exon 10 and utilization of one of the two imperfect polyadenylation termination sites, thereby creating different 3′-untranslated regions. Multiple transcription factors bind to the Axl promoter, including specificity protein 1 and specificity protein 3, myeloid zinc finger 1, and activator protein 1. In cancer, increased Axl expression has been reported at the messenger RNA (mRNA) and protein levels. Transcriptional factors implicated in driving Axl expression include mutant p53, yes-associated protein 1 (in non–small cell lung cancer [NSCLC]), and hypoxia-inducible factor-1 (in renal cell carcinoma).[7–9]

Axl expression is also regulated through various epigenetic mechanisms. Axl promoter hypermethylation results in downregulation of Axl expression. Additionally, Axl mRNA is degraded in the presence of microRNAs mir-34 and mir-199a/b. Methylation status of mir-34 and mir-199a/b correlate with Axl expression and are associated with worse survival in NSCLC.[8] Axl protein folding is dependent on the heat shock protein 90 (HSP90) chaperone such that HSP90 inhibition leads to increased Axl degradation.[10]

Axl gene amplification has been reported in 5% of colorectal cancer tissue samples and has been described

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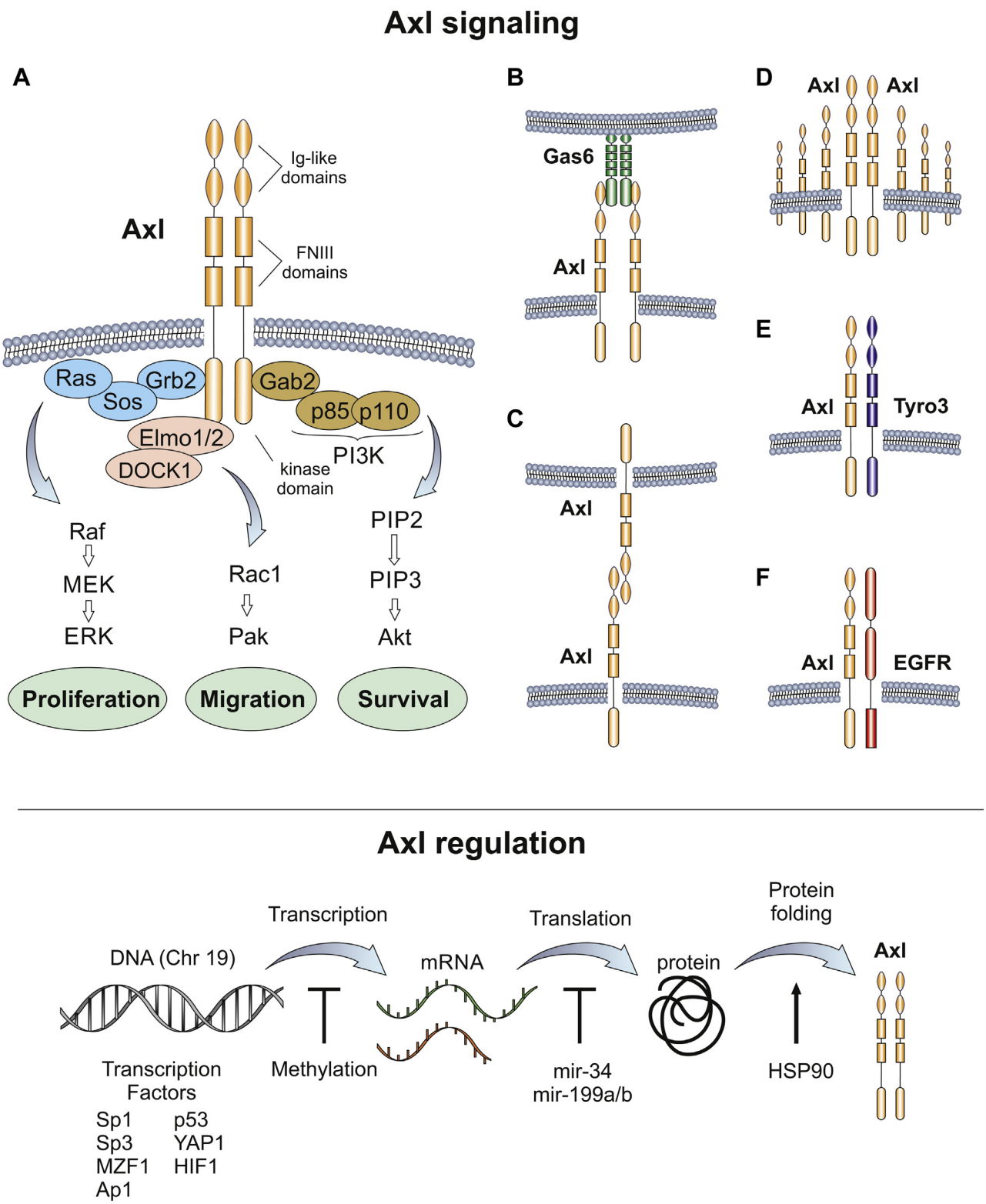

Figure 1. Axl signaling and regulation. *Upper panel*: Axl signlaling. (*A*) Axl signaling includes three well-described pathways: (1) Ras/Raf/MEK/ERK, (2) Elmo1/2/DOCK1/Rac1/Pak, and (3) Gab2/PI3K/Akt. These pathways promote cancer cell proliferation, migration, and survival. (*B-F*) Patterns of Axl activation, including ligand-dependent activation through interaction with Gas6 (*B*) and ligand-independent activation either through Axl transcellular homophilic binding and adhesion (*C*), overexpression (*D*), interaction with another TAM family member receptor (Tyro3) (*E*), or heterodimerization with a non-TAM family receptor such as EGFR (*F*). *Lower panel*: Axl regulation. Axl synthesis from DNA to mRNA to protein is regulated at each step by transcription factor activation, DNA methylation, RNA interference, and protein folding. Ap1, activated protein 1;

in lung adenocarcinoma as well, but the prevalence of *Axl* amplification in other cancer types is poorly characterized.[11,12] Transcriptome sequencing of 200 surgical tumor samples of lung adenocarcinoma revealed a new Axl, MAP3K12-binding inhibitory protein fusion gene (*MBIP*), which preserved the Axl tyrosine kinase domain.[13]

The structure of Axl has been well described. Like other members of the TAM family, the extracellular N-terminal portion of the Axl receptor protein consists of two immunoglobulin domains and two fibronectin type 3 domains linked to a single transmembrane domain. The intracellular portion of the receptor contains conserved kinase domains, including a KWIAIES sequence of amino acids unique to this family of receptor tyrosine kinases.[14]

Axl activation depends on the presence of Gas6 ligand and PS. Evidence suggests that the affinity of Gas6 for Axl is sufficiently high that under physiologic conditions the two may be constitutively bound. However, only in the presence of PS is Gas6 capable of fully activating Axl.[15] Upon activation, Axl homodimerizes and tyrosine amino acids are autophosphorylated at positions 779, 821, and 866. Their phosphorylation creates binding sites for multiple adaptor proteins, including growth factor receptor-bound protein 2 and phospholipase C, which in turn result in activation of key intracellular pathways responsible for proliferation and survival, such as Ras/MEK/ERK and phosphoinositide-3 (PI3K)/protein kinase B.[16,17] Axl can also activate Rac1, leading to cytoskeletal reorganization and a more mesenchymal-like phenotype, characterized by increased migration and invasion.[18]

Role of Axl in Cancer

Axl was initially isolated from chronic myelogenous leukemia cells as a protein capable of transforming the fibrocyte cell line NIH 3T3.[19] Since that time, Axl overexpression has been noted in multiple cancer types, including lung, breast, ovarian, gastric, colon, pancreatic, and prostate. Axl expression and activity are associated with epithelial-to-mesenchymal transition (EMT), higher metastatic potential, therapeutic resistance, and overall worse prognosis.[20–23] In NSCLC and head and neck squamous cell cancer, Axl expression is driven by activation of the EGFR/Ras/mitogen-activated protein kinase pathway and leads to increased resistance to anti-EGFR therapy.[24] Moreover, in head and neck cancer and esophageal squamous cell cancer, Axl was shown to bind and activate EGFR receptor in a ligand-independent manner. This interaction leads to activation of protein kinase C and downstream mammalian target of rapamycin independently from PI3K-α, thereby rendering tumor cells resistant to PI3K inhibitors.[4] Conversely, Axl inhibition sensitizes resistant cells to cytotoxic agents and to targeted inhibitors across cancer types. In head and neck squamous cell cancer, Axl inhibition decreases cancer cell proliferation, migration, and invasion and increases sensitivity to the anti-EGFR antibody cetuximab.[25] In a breast cancer model, sensitivity to taxanes increased 1000-fold with coincident Axl inhibition.[26] Axl is also expressed on endothelial cells and plays a role in angiogenesis. Preclinical studies have shown that Axl inhibition decreases endothelial cell tube formation in vitro and potentiates the antiangiogenic effect of anti–vascular endothelial growth factor antibodies.[27]

Axl Pathway in Lung Cancer

In lung cancer cell lines, Axl mRNA and protein expression correlate with increased migration and invasion, which can be inhibited with Axl small interfering RNA. In clinical specimens, Axl is expressed in approximately half of lung cancer cases and is associated with lymph node involvement, more advanced clinical stage, and worse survival.[28] The association between Gas6 expression and prognosis is less clear. Gas6 protein expression by immunohistochemical analysis is associated with worse clinical outcomes. However, Gas6 mRNA expression—which is inversely related to protein expression—confers improved 5-year survival.[29] This discordance may reflect the fact that most ligand expression comes from nonmalignant cells (such as endothelium, fibroblasts, and immune cells) and may not be related to cancer cell expression of Gas6.

In lung cancer, Axl has been associated with drug resistance. Analysis of tissue samples from patients who had activating epidermal growth factor receptor gene (*EGFR*) mutations and in whom resistance to EGFR inhibitors subsequently developed revealed that almost 30% of previously unexplained mechanisms of resistance to EGFR inhibition—that is, without evidence of a characteristic molecular phenotype such as *EGFR* T790M

Chr 19, chromosome 19; DOCK1, dedicator of cytokinesis 1; EGFR, epithelial growth factor receptor; Elmo 1/2, engulfment and cell motility protein 1 and 2; ERK, extracellular signal regulated kinase; FNIII, fibronectin III; Gab2, GRB2-associated binding protein 2; Gas6, growth arrest-specific 6; Grb2, growth factor receptor-bound protein 2; HIF1, hypoxia-inducible factor 1; HSP90, heat shock protein 90; Ig, immunoglobulin; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MZF1, myeloid zinc finger 1; PAK, p21 protein-activated kinase; PI3K, phosphoinositide-3 kinase (consists of p85 and p110 subunits); PIP_2, phosphatidylinositol (3,4)-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-triphosphate; Rac1, Rho-family small GTP-binding protein 1; SOS, son of sevenless; SP1 and SP3, specificity protein 1 and 3; YAP1, yes-associated protein 1.

mutation or MMNG HOS Transforming gene (*MET*) amplification—could be attributed to EMT.[30,31] Screening of tumor samples and 54 different NSCLC cell lines for an EMT gene signature identified *Axl* expression (protein and mRNA) as highly correlated with mesenchymal phenotype. Analysis of cancer tissues from patients with NSCLC has demonstrated that Axl overexpression can coexist with *EGFR* T790M mutation and that these two mechanisms of resistance are not mutually exclusive.[30] In cell lines and xenograft models, administration of the Axl tyrosine kinase inhibitor SGI-7079 reversed mesenchymal phenotype and increased EGFR inhibitor sensitivity.[20] Similar sensitization to anti-EGFR therapy was also achieved by promoting Axl degradation through inhibition of HSP90.[10] A key question related to the role of Axl inhibition in the approach to *EGFR*-mutant NSCLC is whether it would be more advantageous to use such combinations upfront to preempt resistance or to reserve the use of Axl inhibitors for the purpose of reversing resistance after it develops. Both strategies are being investigated in early clinical trials.

In some lung cancers, *Axl* alterations may render tumors highly sensitive to Axl inhibition. For instance, a patient with heavily pretreated advanced NSCLC harboring *Axl* amplification experienced a partial response to the single-agent MET proto-oncogene/Axl inhibitor MGCD265, with a 48% reduction in tumor dimension and substantial symptomatic improvement.[11]

Additionally, Axl and other TAM receptors have immunomodulatory effects. Activation of Axl in immune cells suppresses proinflammatory cytokine release and decreases inflammation. Therefore, Axl-mediated signaling may aid in creation of an immunotolerant milieu and allow tumor growth. Preclinical studies have shown that targeted inhibition of Axl or its downstream signaling in natural killer cells promotes anticancer immune responses in melanoma and breast cancer mouse models.[32] These properties suggest that Axl inhibition might be incorporated into existing cancer immunotherapy strategies to augment treatment efficacy.

Development of Axl Inhibitors in Lung Cancer

To date, most clinical studies of Axl inhibition in lung cancer have used nonspecific multikinase inhibitors, which target Axl among a number of receptor tyrosine kinases (Table 1). The small molecule inhibitor cabozantinib has been approved for treatment of medullary thyroid cancer because of its anti-RET activity. Cabozantinib also has activity against vascular endothelial growth factor receptor, MET, Flt3, Kit, and Axl. There are several ongoing clinical trials investigating the benefit on cabozantinib in NSCLC either as monotherapy or in combination with erlotinib. In addition to its well-known effects on anaplastic receptor tyrosine kinase, MET, and Ros proto-oncogene 1, receptor tyrosine kinase, crizotinib also targets Axl. Other multitargeted kinase inhibitors with Axl activity include ASLAN002, MGCD265, MGCD516, and foretinib. Recently, a number of more specific Axl inhibitors such as BGB324 and BPI-9016M have entered early-phase clinical trials.[33] The anti-Axl monoclonal antibody YW327.6S2 and Axl decoy receptor and aptamer GL2I.T are undergoing preclinical development.[34,35] For Axl pathway targeting, small molecule and antibody-based approaches have distinct characteristics and advantages. Although antibodies as a class are generally more specific than small molecules, the effect of these drugs will depend on the selected target.

Table 1. Axl Inhibitors in Clinical Trials

Tyrosine Kinase Inhibitor	Target Protein	Axl IC_{50}	Clinical Trial (Disease) (Combination)	Phase
Cabozantinib	Axl, MET, VEGFR2, RET, Kit, Flt-1, Flt-3, Flt-4, Tie2	7 nM	NCT01639508 (NSCLC) NCT00596648 (NSCLC) (+ erlotinib) NCT01708954 (NSCLC) (+ erlotinib) NCT01866410 (NSCLC) (+ erlotinib)	2 1 and 2 2 2
Crizotinib	Axl, Alk, MET, RON, ROS1	294 nM	NCT02034981 (multiple cancers)	2
ASLAN002	Axl, RON, MET, Tyro3, Mer, Flt-3	1.1 nM	NCT01721148 (multiple cancers)	1
MGCD265	Axl, MET, VEGFR2		NCT00697632 (multiple cancers)	1
MGCD516	Axl, RET, TRK, DDR2, MET, Kit, VEGFR, PDGFR		NCT02219711 (multiple cancers)	1/1B
Foretinib + erlotinib	Axl, MET, VEGFR2, RON	11 nM	NCT00725764 (HNSCC) NCT01068587 (NSCLC) (+ erlotinib)	2 1 and 2
BGB324	Axl	14 nM	NCT02488408 (AML) (+ cytarabine) NCT02424617 (NSCLC) (+ erlotinib)	1 1 and 2
BPI-9016	Axl, MET		NCT02478866 (multiple cancers)	1

AML, acute myeloid leukemia; Alk, anaplastic lymphoma kinase; DDR2, discoidin domain receptor 2; Flt-1, Flt-3, and Flt-4, FMS-related tyrosine kinase 1, 3 and 4; HNSCC, head and neck squamous cell cancer; IC_{50}, concentration that inhibits 50%; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; RET, ret proto-oncogene; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase; Tie2, epithelial-specific protein receptor tyrosine kinase; TRK, tyrosine kinase receptor; VEGFR2, vascular endothelial growth factor receptor 2; RON, RON protein tyrosine kinase; MER, MET proto-oncogene.

Although anti-Gas6 antibodies should inhibit only ligand-induced Axl activation, anti-Axl antibodies and small molecule inhibitors should inhibit ligand-dependent and ligand-independent activity. Finally, because Gas6 binding to PS requires vitamin K–dependent y-carboxylation of the γ-carboxyglutamic domain, the vitamin K antagonist warfarin has been shown to inhibit TAM receptor signaling and reduce malignant potential.[36] Further investigation is needed to determine the clinical benefit of warfarin as a cancer therapeutic agent.

Conclusion

Growing evidence suggests that Axl is critical for cancer cell survival, EMT, metastatic potential, and therapeutic resistance in many cancer types, including NSCLC. A number of clinically available multitargeted kinase inhibitors, such as cabozantinib and crizotinib, inhibit Axl function. Recently, a number of more specific Axl kinase inhibitors have entered early-phase clinical trials, and anti-Axl monoclonal antibodies and decoy receptors are undergoing preclinical development. Results from these studies will give important insights into the potential of Axl targeting as a therapeutic approach, especially for patients with de novo or acquired resistance to existing targeted therapies.

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