Activation of TYRO3/AXL Tyrosine Kinase Receptors in Thyroid Cancer

Elvira Avilla¹, Valentina Guarino¹, Carla Visciano¹, Federica Liotti¹, Maria Svelto¹,³, GnanaPrakash Krishnamoorthy¹, Renato Franco³, and Rosa Marina Melillo¹,²

Abstract

Thyroid cancer is the most common endocrine cancer, but its key oncogenic drivers remain undefined. In this study we identified the TYRO3 and AXL receptor tyrosine kinases as transcriptional targets of the chemokine CXCL12/SDF-1 in CXCR4-expressing thyroid cancer cells. Both receptors were constitutively expressed in thyroid cancer cell lines but not normal thyroid cells. AXL displayed high levels of tyrosine phosphorylation in most cancer cell lines due to constitutive expression of its ligand GAS6. In human thyroid carcinoma specimens, but not in normal thyroid tissues, AXL and GAS6 were often coexpressed. In cell lines expressing both receptors and ligand, blocking each receptor or ligand dramatically affected cell viability and decreased resistance to apoptotic stimuli. Stimulation of GAS6-negative cancer cells with GAS6 increased their proliferation and survival. Similarly, siRNA-mediated silencing of AXL inhibited cancer cell viability, invasiveness, and growth of tumor xenografts in nude mice. Our findings suggest that a TYRO3/AXL-GAS6 autocrine circuit sustains the malignant features of thyroid cancer cells and that targeting the circuit could offer a novel therapeutic approach in this cancer. Cancer Res; 71(5); 1792–804. ©2011 AACR.

Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence is increasing worldwide. Thyroid cancer histotypes include: well-differentiated papillary and follicular (PTC and FTC), poorly differentiated (PDTc) and anaplastic thyroid carcinoma (ATC; ref 1, 2, 3). Thyroid cancer features overexpression of specific chemokine and their receptors (4, 5). CXCR4/SDF-1 axis has an important role in promoting cell growth, invasiveness, and survival in thyroid cancer cells and its blockade can revert all these phenotypes (4, 5, 6). We analyzed global gene expression profiles of CXCR4-expressing human PTC cells (TPC-1) with or without SDF-1α. We identified TYRO3 and AXL, belonging to the TAM family (Tyro3, Axl, and Mer) of tyrosine kinase receptors (7, 8). TAM-mediated signaling is involved in cell survival, proliferation, migration and adhesion (9), vascular smooth muscle homeostasis (10), platelet function (11; 12), and erythropoiesis (11). These receptors are frequently coexpressed in vascular, reproductive, nervous, and immune system in adults (13). TAM receptors can be activated by 2 physiological ligands, GAS6 (Growth-Arrest-Specific gene 6) and Protein S, which are homologous vitamin-K-dependent proteins (13). TAM receptors are involved in cancer development and progression. AXL overexpression is observed in many human cancer types. Gas6 expression is frequently expressed in cancer and its level correlates with poor prognosis (14).

The aim of this report is to study TAM receptor involvement in thyroid cancer. We show that human PTC/ATC cells and samples, but not normal thyroid, constitutively express AXL, TYRO3, and their ligand GAS6. We show that AXL, TYRO3, and GAS6 have a critical role in mediating thyroid cancer cell proliferation, invasiveness, and survival. Moreover, we show that silencing of AXL in an ATC cell line strongly affects tumor growth in immunodeficient mice. These data provide evidence that the AXL/TYRO3/GAS6 axis might be exploited as target for potential antitumoral therapy.

Materials and Methods

Cell cultures

Human primary cultures of normal thyroid were obtained from F. Curcio (Dipartimento di Patologia e Medicina Sperimentale e Clinica, University of Udine, Italy) and cultured as described (15). Human thyroid papillary cancer cell lines TPC1, BCPAP, NIM, and anaplastic thyroid cancer cell lines 850–5C, CAL62, U-HTH83, C643, SW1736, U-HTH7, U-HTH83, C643, SW1736, OCUT-1, ACT-1 have been previously described (15–17). PC, PC BRAF, PC HaPTC3, PC E1A, PC PTC1, PC E1A-RAF, PC PTC3, PC v-mos, PC E1A-RAF rat thyroid epithelial cells have been previously described (4, 5, 18). Transformed human embryonic kidney cells (293T) have been obtained by ATCC (19). TPC1 and NIM were obtained from Dr. M. Santoro.
(University of Naples, Naples, Italy); BCPAP, CAL62, and 850-5C were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. OCUT-1 and ACT-1 were a gift of K. Hakawara and N. Onoda (Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan) and SW1736, C643, U-HHTH7, and U-HTH83 cells were a gift of C.H. Helden (Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden). All cells have been tested for specific mutations and thyroid marker expression. Cells are passaged for fewer than 3 months.

**Histology and immunohistochemistry**

Thyroid tissue samples from patients affected by thyroid carcinomas and normal thyroid tissues were obtained from the Struttura Complessa di Anatomia Patologica, Istituto Nazionale Tumori di Naples, upon informed consent. Anti-AXL, anti-GAS6, and anti-CXCR4 antibodies from R&D Systems were used to stain human thyroid carcinoma samples. For xenograft hystological analysis, Ki67 antibody (Clone MIB-1) was from Dako, and CD31 antibody was from Santa Cruz Biotechnology; samples were processed with peroxidase detection system reagent kit (Novocastra). Apoptosis was evaluated by the terminal deoxynucleotidyl transferase (TdT)-mediated dNTP-labeling (TUNEL) method using Fragel kits. Sections were deparaffinized and rehydrated and antigen retrieval procedures were performed with recombinant human GAS6 (200ng/mL) for 24 hours. BrdU was selected in medium with 500ng/mL puromycin. To block AXL, we also generated 850-5C cells that stably express dominant-negative forms of the receptor, that is FLAG-tagged AXL EC, that lacks the whole intracellular domain, and the kinase dead mutant of AXL carrying the mutation K558R, which abrogates TK activity (Myc-tagged AXL KD). Detailed experimental procedures regarding these mutants are included in Supplementary data.

**Protein studies**

Protein extractions, immunoblotting, immunoprecipitation, and pull-down binding assays were carried out according to standard procedures. Anti-AXL were from Santa Cruz Biotechnology. Anti-TYRO3 was from R&D Systems. Anti-phospho AXL, specific for tyrosine 779, was from R&D Systems. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology. Anti-AKT and anti-phospho-AKT were from Cell Signaling. Anti-phospho-p44/42 MAPK and anti-p44/42 MAPK were from Cell Signaling. Anti-phospho-p70S6K and anti-p70S6K were from Cell Signaling. Monoclonal anti-tubulin was from Sigma Chemical Co. Anti-GRB2 antibodies were from Santa Cruz Biotechnology; anti-p85 was from Cell Signaling. Secondary anti-mouse, anti-goat, and anti-rabbit antibodies coupled to horseradish peroxidase were from Bio-Rad. Detailed experimental procedures can be found in Supplementary data.

**ELISA assay**

Thyroid cells were allowed to grow 70% confluency and serum-deprived for 12 hours. GAS6 levels in culture supernatants were measured using a quantitative immunoassay ELISA kit (DuoSet ELISA Development Kit, R&D Systems). Samples in triplicate were analyzed at 450 nm with an ELISA reader (Model 550 microplate reader, Bio-Rad).

**Matrigel invasion**

*In vitro* invasiveness through Matrigel was assayed using transwell cell culture chambers according to described procedures (4). Cells (1 × 10^5 cells/well) were added to the upper chamber of a prehydrated polycarbonate membrane filter of 8 μm pore size (Costar, Cambridge) coated with Matrigel (Collaborative Research) and then incubated at 37°C in a humidified incubator in 5% CO2 and 95% air for 24 hours. Migrating cells on the reverse side of the filter were stained with 0.1% crystal violet in 20% methanol for 30 minutes, lysed in 10% acetic acid, and analyzed at 570 nmol/L with an ELISA reader (Model 550 microplate reader, Bio-Rad). The results were expressed as percentage of migrating cells with respect to the sh-CTR silenced ones.

**Cell proliferation**

S-phase entry was evaluated by 5-bromo-2-deoxyuridine (BrdU) incorporation and indirect immunofluorescence according to described procedures (4, 5). Cells were grown on coverslips, serum-starved for 12 hours, and treated with recombinant human GAS6 (200ng/mL) for 24 hours. Moreover, cells were silenced with siRNA (Dharmacon) for 24 hours, serum-deprived for 12 hours, and then treated with recombinant human GAS6 (200ng/mL) for 24 hours. BrdU was
added at a concentration of 10 μmol/L and BrdU-positive cells were revealed with Texas-Red–conjugated secondary antibodies (Jackson Immuno Research Laboratories).

For growth curves, cells were plated at a density of $0.5 \times 10^5$ in low serum conditions (2.5%) and counted at the indicated time-points.

**Flow cytometric analysis (FACS)**

Cells were transfected with control siRNA (siCTR) or AXL-targeting siRNAs (siAXL) and 48 hours later detached from culture dishes with a solution of 0.5 mmol/L EDTA. After saturation with 1 μg of human IgG/10^5 cells, cells were incubated for 20 minutes on ice with antibodies against AXL extracellular domain (R&D Systems) or isotype control antibody. Unreacted antibody was removed and cells were incubated with phycoerytrin-labeled secondary antibodies (R&D Systems). Cells resuspended in PBS were analyzed on a FACS-Calibur cytometer using the CellQuest software (Becton Dickinson). Analyses were done in triplicate. In each analysis, a total of $10^4$ events were calculated.

**TUNEL assay**

For TUNEL, an equal number ($5 \times 10^5$) of cells was seeded onto single-well Costar glass slides; after 24 hours of silencing, cells were serum-deprived for 12 hours and treated with GAS6 (200 ng/mL) for 24 hours, and subjected to the TUNEL reaction (Roche) as described elsewhere (4).

**Xenografts in nude mice**

Each group of 10 mice (4-week-old male BALB/c nu/nu mice, Jackson Laboratories) was inoculated subcutaneously into the right dorsal portion with 850-5C shCTR cells, 850-5C shAXL C12, 850-5C shAXL C14, or 850-5C shAXL C16 (10 x $10^6$/ mouse). Tumor diameters were measured at regular intervals with the caliper. Tumor volumes (V) were calculated with the formula: $V = A \times B^2/2$ ($A =$ axial diameter; $B =$ rotational diameter). This study was conducted in accordance with Italian regulations for experimentation on animals.

**Statistical analysis**

For comparisons between cell lines, we used the parametric 2-tailed t-test. To calculate the association between CXCRI4 and AXL expression in PTCs, we used the 2-sided Fisher’s Exact Test, with the GraphPad Instat software, v.3.0b. Differences were statistically significant at $P < 0.05$.

**Results**

**TYRO3 and AXL receptors are overexpressed and activated in thyroid cancer cells**

We carried out global gene expression profiling of human TPC1 cells with or without SDF-1c. Among several genes, TYRO3 and AXL were significantly upregulated. Quantitative RT-PCR on selected genes and Western blot analysis on TYRO3 and AXL confirmed these findings (Supplementary Fig. S1A and B). Treatment of thyroid cancer cell lines with the CXCRI4 inhibitor AMD3100 inhibited SDF-1-mediated AXL upregulation (data not shown), but not constitutive AXL expression, indicating that other mechanisms are responsible for this phenomenon. Thyroid cancer cell lines carry activated oncogenes belonging to the RAS-BRAF-ERK, the p53 or the PI3K/AKT pathway. TYRO3 and AXL protein levels were undetectable in normal thyroid cells, whereas they were significantly expressed in cancer cell lines (Fig. 1A). This was also confirmed by RT-PCR experiments (Supplementary Fig. S1C). AXL is activated in different cancer histotypes (21–25). Thus, we evaluated its tyrosine-phosphorylation levels in our cell systems by using both anti- phosphotyrosine and phospho-Tyr 779 (Y779) antibodies. Cancer cells displayed different degree of AXL tyrosine-phosphorylation (Fig. 1A). TPC1 cells, derived from a human PTC featuring a spontaneous RET/PTC1 rearrangement, showed the highest levels. Many thyroid cancer cell lines (850-5C, NIM, and CAL62) expressed the AXL ligand GAS6, but not TPC1 (Fig. 1B and Supplementary Fig. S1C). AXL was also overexpressed in most of the oncogene-transfected rat thyroid cells lines (PC CI3) but not in PC E1A and PC BRAF (Fig. 1C). Again, PC PTC cells show the highest level of AXL phosphorylation (Fig. 1C). These data suggest that RET/PTC may be involved in AXL activation through a ligand-independent mechanism, whereas other genetic lesions induce a ligand-dependent activation of the receptor. AXL, through a C-terminal multidocking tyrosine (Y821), binds to several substrates, such as p85 and GRB2. Tyrosine 779 was identified as an additional p85-binding site (26). Although p85 activation invariably results in the activation of the phosphatidylinositol-3-kinase/Akt pathway, GRB2 can signal to both the PI3-K/Akt and the extracellular regulatory kinase (ERK) pathways (27). We analyzed the pattern of AXL-binding proteins in TPC-1 and 850-5C cells by AXL immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies. p85, but not GRB2 (not shown), coprecipitated with AXL (Fig. 1D). However, in p85 immunoprecipitates, we could not detect AXL. This could be due to the interference of p85 antibody with AXL/p85 interaction, or to the limiting amount of endogenous proteins in our system. To distinguish between these possibilities, we overexpressed AXL and p85 in 293T cells and showed the presence of AXL in p85 immunoprecipitates and in GST-SH2 p85 pull-down assays (Supplementary Fig. S2). By using TPC1 and 8505-C cell lysates, AXL binding was detected in N-terminal p85 SH2- (Supplementary Fig. S3), but not GRB2 SH2 (data not shown) pull-down experiments. Consistently, the phosphorylation of AKT, but not of ERK1/2, was observed upon GAS6 stimulation of thyroid cancer cells (Supplementary Fig. S3). We also overexpressed AXL and GRB2 in 293T cells. In this experiment, we detected AXL-GRB2 coimmunoprecipitation, but GRB2 binding was not observed in pull-down assays (Supplementary Fig. S2). Taken together, our data indicate that AXL can bind to both p85 and GRB2. However, in thyroid cancer cells, it preferentially binds to p85.

**Human thyroid cancer specimens express Axl and Gas6**

Twenty-seven thyroid carcinoma samples (9 PTCs, 10 FTCs, and 8 PDC/ATCs) were analyzed by immunohistochemical staining with an anti-human AXL antibody. Of all the samples
Figure 1. A, analysis of TYRO3 and AXL protein levels and AXL tyrosine-phosphorylation in a panel of human PTC and ATC cells compared with the total AXL protein levels. Anti-tubulin monoclonal antibody was used as a loading control. B, evaluation of GAS6 levels in thyroid carcinoma cells by ELISA and Western blot. C, analysis of AXL levels and tyrosine-phosphorylation by AXL-immunoprecipitation followed by Western blot with anti-AXL and anti-phosphotyrosine (PY) in a panel of parental (PC) and transformed PC Cl3 rat thyroid cells. D, lysates from TPC1 and 850–5C cells were immunoprecipitated with anti-AXL and anti-p85 antibodies followed by Western blot with anti-phosphotyrosine, anti-AXL, and anti-p85 antibodies.
analyzed, 73% (19/27) scored positive for AXL expression. Normal thyroid was negative for AXL expression (Supplementary Table 1). AXL positivity was observed mainly in tumoral cells (Fig. 2A). Most of the samples displayed membrane positivity, but a small number of specimens showed cytosolic staining into perinuclear vesicles probably representing the Golgi apparatus, a typical pattern of overexpressed proteins. Tumoral stroma and nontumoral adjacent tissues were negative, with the exception of red blood cells that were strongly recognized by anti-AXL antibody as previously described (28).

We also assessed GAS6 expression by IHC in the same sample set. Most of the analyzed specimens scored positive for GAS6 (Fig. 2A). GAS6 staining was cytosolic and mainly found in carcinoma cells; some samples also displayed stromal positivity, suggesting that the ligand can also be provided by the tumor microenvironment. As AXL was identified as a CXCR4-SDF-1 target, we analyzed an independent set of PTCs (n = 30) for both AXL and CXCR4 immunostaining (Fig. 2B). All the CXCR4-positive samples (10/30) also coexpressed AXL (10/10). AXL was expressed in 14 out of 30 samples. Thus, CXCR4 expression positively correlated with AXL immunostaining (P < 0.005).

**AXL/TYRO3-GAS6 blockade inhibits thyroid cancer cell proliferation and survival**

We analyzed the level of DNA synthesis by BrdU incorporation assays in serum-deprived TPC1 cells with and without GAS6. Upon GAS6 stimulation, BrdU incorporation increased from 10% to 20%. Consistently, a chimaeric protein (Dtk/FC) that contains the extracellular domain of TYRO3 fused to the FC portion of the IgG that sequesters GAS6, did not modify the rate of BrdU-positive cells in basal conditions, but inhibited the effects of GAS6 treatment (Supplementary Fig. S4A). Then, we used RNA interference. AXL silencing was verified by Western blot (Fig. 3A) and FACS analysis (Supplementary Fig. S5). When AXL was silenced, we observed a reduction of BrdU incorporation both in basal conditions (Fig. 3A) and in the presence of GAS6: BrdU-positive cell rate dropped from 27% to 8%. GAS6 silencing did not modify TPC1 growth rate (not shown), consistent with the observation that GAS6 is not produced by these cells. To evaluate whether AXL blockade influenced TPC1 survival, we treated these cells with a proapoptotic substance, diethylmaleate, for 12 hours, and carried out TUNEL assays. In this condition, 48% of TUNEL-positive cells were observed; GAS6 addition dramatically decreased...
Figure 3. A, BrdU incorporation and TUNEL assay were evaluated in TPC1 cells after AXL and/or TYRO3 silencing in presence and in absence of GAS6. Western blot analysis to verify AXL and TYRO3 silencing is shown. Three independent experiments were carried out in which at least 400 cells were counted. B, BrdU incorporation and TUNEL assay of NIM cells treated or not with GAS6 and after AXL, GAS6, and TYRO3 silencing. Western blot analysis to verify AXL, GAS6, and TYRO3 silencing is shown. C, BrdU incorporation and TUNEL assay of 850–5C cells with and without GAS6 and after AXL silencing. Western blot analysis to verify AXL silencing is shown.
this percentage. Dtk/FC decreased the percentage of apoptotic cells only in the presence of exogenous GAS6 (Supplementary Fig. S4B). We also evaluated apoptosis in AXL-silenced cells. After 12 hours serum deprivation, 10% of apoptotic cells were observed. This percentage decreased to 5% when GAS6 was added. AXL silencing dramatically increased the rate of apoptotic cells to 30%. Interestingly, when GAS6 was added to AXL-silenced cells, the proapoptotic effects of AXL silencing were significantly inhibited (Fig. 3A). This effect could be due to incomplete AXL silencing. However, FACS analysis showed that RNAi almost completely suppressed AXL expression (Supplementary Fig. S5). Alternatively, TYRO3 can substitute AXL function. GAS6 silencing did not modify the TPC1 cell apoptotic rate (not shown). As the addition of GAS6 partially recovered the effects of AXL blockade in this cell line, we evaluated the effects of TYRO3 knockdown in TPC1 cells through RNA interference. As shown in Figure 3A, TYRO3 silencing affected both BrdU incorporation and apoptosis. Silencing of both receptors increased the effect of the single knockouts; finally, GAS6 treatment did not modify the effect of the double knockouts. Similar experiments were carried out by using NIM cells that express both AXL and GAS6. Exogenous addition of this ligand did not significantly modify BrdU incorporation and apoptosis rate of NIM cells. Instead, the blockade of both endogenous AXL and GAS6 by using RNAi or Dtk/FC inhibited NIM DNA synthesis, and exogenous GAS6 addition reverted these effects. NIM apoptosis induced by diethylmaleate or serum deprivation was only slightly modified by GAS6 treatment. However, Dtk/FC or AXL- or GAS6-silencing strongly enhanced NIM apoptotic rate, and these effects were again reverted by exogenous GAS6 (Fig. 3B and Supplementary Fig. S4). TYRO3 silencing also affected NIM proliferation and survival, and TYRO3/AXL silencing was as effective as GAS6. GAS6 treatment did not modify the effects of TYRO3/AXL silencing. GAS6, TYRO3, and AXL knockdown was verified by Western blot analysis (Fig. 3B). We also used siRNA to verify whether AXL knockdown could impair proliferation and survival of human 850–5C and CaI62 ATC cells. GAS6 stimulated cell proliferation and inhibited apoptosis both in 850–5C and in CAI62 cells and AXL silencing had the opposite effect (Fig. 3C, Supplementary Fig. S6).

**Effects of stable silencing of AXL in 8505-C ATC cells**

We selected 850–5C cells for their ability to induce tumor formation in immunodeficient mice. We stably transfected these cells with a pool of vectors expressing 5 different shRNAs directed against AXL or expressing control nontargeting shRNAs. We identified clones that expressed intermediate (shAXL Cl 1 and 2) or low (shAXL Cl 3–6) AXL levels (Fig. 4A). These results were confirmed by FACS analysis (Fig. 4B). In 850–5C shAXL clones, the activation of the PI3-K pathway (phosphorylation of Akt and of p70S6 Kinase), was significantly reduced with respect to control or parental cells (Fig. 4C). In contrast, the phosphorylation of ERK1/2 was not affected by AXL silencing. We then verified whether AXL knockdown could impair cell growth both in optimal (10% FCS) or in low serum (2.5% FCS) conditions. AXL silencing showed mild effects on cell growth in complete culture medium (Supplementary Fig. S7), whereas it was significantly more effective at low serum concentration (Fig. 5A). To assess whether reduced cell growth was due to decreased DNA synthesis or increased cell death, cells were serum starved for 12 hours and the percentage of BrdU- and TUNEL-positive cells was evaluated. All shAXL clones displayed a significant reduction of BrdU incorporation and a significant increase in
apoptosis when compared with control clones, and these effects were partially reverted by exogenous GAS6 (Fig. 5B). Moreover, we asked whether AXL was necessary for ATC cell invasiveness through Matrigel both in the absence and in the presence of SDF-1α. shAXL clones showed a clear decrease of invasive ability with respect to shCTR cells (Fig. 5C). SDF-1α stimulated migration of cells displaying residual AXL expression, but was unable to do so in the AXL-negative clones, suggesting that AXL is required for SDF-1α-mediated cell invasion of 850-5C cells. To rule out unspecific effects of the AXL shRNAs, we decided to inhibit AXL through an alternative strategy. We generated 850-5C cells expressing dominant-negative AXL proteins, a truncated form of human AXL, lacking the intracellular domain (AXL-EC), or a kinase-dead mutant, carrying the substitution K558R (AXL KD). Phospho-tyrosine blotting confirmed inhibition of AXL signaling (Fig. 6A). AXL-EC and AXL KD inhibited AXL-mediated DNA synthesis, cell survival, and cell migration (Fig. 6B, C, and D).

Figure 5. A, growth curves of 850–5C shCTR and shAXL clones done in 2.5% FBS. The average results of at least 3 independent determinations were reported. B, BrdU incorporation was measured to evaluate S-phase entry of 850–5C shAXL and shCTR clones both in the absence and in the presence of exogenous GAS6. TUNEL reaction was conducted on 850–5C shAXL and shCTR clones both in the presence and in the absence of exogenous GAS6. C, Matrigel invasion assay on 850–5C shAXL clones. The results were expressed as percentage of 850–5C shAXL migrated cells with respect to 850–5C shCTR.
**Axil silencing inhibits experimental tumor growth**

Finally, we evaluated the role of AXL in tumor growth by using xenografts of ATC cells into (nu/nu) immunodeficient mice. We injected animals with parental, shCTR-transfected (shCTR), and shAXL-expressing (C4 and Cl6) 850–5C cells, and compared their growth rate. Parental cells formed tumors with the same efficiency as shCTR-transfected cells did (not shown). As shown in Fig. 7A, the tumorigenicity of 850–5C shAXL clones was completely inhibited when compared with 850–5C shCTR control cells. At 3 weeks, shCTR tumor median volume was 34 mm³, whereas shAXL was less than 10 mm³. At 4, 5, and 6 weeks, shCTR tumors continued to grow, reaching a median volume of approximately 150 mm³ whereas shAXL did not, and the few small tumors that appeared at 3 weeks eventually regressed. At the end of the experiment, no shAXL tumors were observed. This precluded histological analysis. We then tested the ability of 850–5C shAXL clones with intermediate levels of AXL expression to induce tumor formation in nude mice. We injected mice with shAXL C2, that still retains some expression of the receptor (Fig. 7B), and, as control, the shCTR clone. A few small tumors appeared at 7 weeks postinjection in the shAXL C2 group (Fig. 7B). At the end of the experiment, tumors were excised and analyzed by IHC for proliferation, apoptosis, and vascularization. A higher rate of apoptosis, but not of proliferation, of shAXL tumors with respect to controls was observed, although differences were not statistically significant due to the limited number of shAXL samples (Fig. 7C). Vessel density was comparable between the 2 groups of tumors. However, in shAXL tumors, vessels were not well-formed and their median diameter was smaller than that of shCTR clones (P < 0.05). These data indicate that AXL is required for 850–5C xenograft growth in nude mice.

**Discussion**

In the search for SDF-1 transcriptional targets in thyroid cancer, we identified 2 tyrosine-kinase receptors, TYRO3 and AXL. AXL overexpression has been reported in a variety of human cancers (21, 24, 28–36), and it is associated with negative prognosis (14, 24, 25, 37–39). AXL expression confers resistance to different aneoplastic agents (40–42), can induce angiogenesis (14, 43), cancer cell migration and invasion (37, 44–46).

We found that TYRO3 and AXL are transcriptionally regulated by CXCR4-SDF-1. However, these receptors wereconstitutively expressed in human thyroid cancer cells, but not in normal thyroid. AXL expression was also detected in thyroid cancer samples, but not in normal thyroid tissues, and the presence of AXL positively correlated with CXCR4 expression. Rat thyroid cancer cells (PC) expressing different activated oncogenes also expressed AXL, with the exception of PC BRAF and PC E1A. These data indicate that multiple mechanisms, besides CXCR4 activation, can contribute to increase AXL levels in thyroid cancer. The activation of components of the RAS/MAPK pathway is frequently found in human PTC. However PC PTC, but not PC BRAF cells, express AXL, despite both display activated MAPKs. This suggests that the RAS/MAPK pathway is not sufficient to activate AXL expression.

Cancer-associated transcription factors, promoter hypomethylation or gene amplification may result in AXL overexpression. Interestingly, AXL is strongly induced by epithelial-to-mesenchimal transition (37) or by a v-FLIP-NFKB pathway (47). Preliminary data obtained by us failed to show gene mutation or amplification in thyroid cancer cell lines (Krishnamoorthy, unpublished observations). However, other possible mechanisms of AXL expression in thyroid cancer need to be investigated. AXL was constitutively phosphorylated in most of the human thyroid cancer cells. This was due to the presence of the ligand GAS6; the only exception was the TPC1 cell line, in which AXL was highly phosphorylated despite ligand absence. In this case, some other mechanisms, such as Ret-mediated ligand-independent activation of AXL, may keep the receptor in a constitutively active status. Consistently, a cross-talk between EGFR family members and AXL has been suggested (38, 42). The inhibition of TYRO3, AXL, or GAS6 reduced cell proliferation and increased apoptotic rate. AXL silencing in 850–5C ATC cells dramatically reduced thyroid cancer cell invasive ability, and SDF-1 could not rescue this capability, indicating that AXL is required for SDF-1-mediated chemotactic activity. These results are in accord with previous data indicating that AXL is involved in cell migration and invasion (37, 44). AXL is also involved in angiogenesis (14, 43). Accordingly, in our xenograft system, AXL silencing strongly inhibited 850–5C tumor formation, and this effect was mainly due to impaired vessel formation in xenografts. We also observed increased apoptosis in 850–5C shAXL induced tumors with respect to the normal counterpart. However, differences in the apoptotic rate were not statistically significant. Thus, in our thyroid cancer model, AXL seems to be required for the initial steps of tumor formation. Other studies have shown that AXL is required for breast cancer cell xenograft growth in nude mice, but it was not required for orthotopic breast tumor formation. However, it was necessary for invasion and metastasis (37). Differences in AXL requirement between subcutaneously- or orthotopically-injected breast tumors suggested a specific function for this receptor to drive growth and colonization in an unfavorable environment (48, 49). Whether orthotopic thyroid tumor formation in syngeneic animals requires AXL is currently unknown. In breast cancer, AXL expression has been associated with the presence of metastasis and it represents a negative prognostic factor (37). We have analyzed a limited number (n = 27) of thyroid cancer samples, ranging from the well-differentiated PTC and FTC to the completely undifferentiated ATC, and we found that AXL expression was equally distributed in all 3 histotypes. This analysis, although done on a small number of patients, indicates that AXL expression may be an initial event of thyroid transformation, but it is sometimestained during thyroid cancer progression. TYRO3, often coexpressed with AXL in thyroid cancer cells, is also important for thyroid cancer cell viability. However, tumorigenesis in nude mice is strictly dependent on AXL.

In conclusion, our data strongly suggest that AXL/TYRO3-GAS6 axis can be considered as a novel potential target of thyroid anticancer therapy. Several compounds have been identified that block AXL signaling by acting at different levels.
Figure 6. A, schematic representation of wt and mutant AXL. AXL-EC includes aa 1 to 508, whereas AXL KD contains the substitution K558R. FLAG and myc-his epitopes are indicated. Exogenous AXL KD and AXL EC expression in different clones was verified by Western blot analysis with anti-myc and anti-FLAG antibodies, respectively. 850–5C AXL KD cl 1 and AXL EC MP1 were also analyzed for AXL phosphorylation through anti-phospho-AXL immunoblot. B, Matrigel invasion assay on control (pcDNA3.1), AXL KD- and AXL EC- 850–5C cells. The results were expressed as the percentage of migrating cells with respect to controls. C, BrdU incorporation and TUNEL assays were carried out to evaluate S-phase entry and apoptosis of 850–5C (NT), empty vector-transfected (pcDNA3.1), and AXL mutant clones. The percentage of apoptotic cells was measured by TUNEL assay in 850–5C (NT), empty vector-transfected (pcDNA3.1), and AXL mutant clones. NT, normal tissue.
Figure 7. A, three groups of 10 nude mice injected with 850–5C shCTR or 850–5C shAXL CL4 or CL6 cells were evaluated for tumor growth. All P values were 2-sided, and differences were statistically significant at P < 0.05. B, 2 groups of 10 nude mice injected with 850–5C shCTR cells or 850–5C shAXL Cl2 that expressed intermediate levels of AXL were evaluated for tumor growth. Mice inoculated with shAXL Cl2 formed tumors after 7 weeks, but their size was smaller than that of shCTR tumors. C, IHC on excised shAXL Cl2 and shCTR tumors for ki67, CD31, and Tunel. Mean vessel diameter was significantly lower in shAXL than in control clones; *P < 0.02.
Our data indicate that these compounds should be exploited to treat thyroid cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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