AXL Is a Key Regulator of Inherent and Chemotherapy-Induced Invasion and Predicts a Poor Clinical Outcome in Early-Stage Colon Cancer

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Abstract

Purpose: Despite the use of 5-fluorouracil (5-FU)–based adjuvant treatments, a large proportion of patients with high-risk stage II/III colorectal cancer will relapse. Thus, novel therapeutic strategies are needed for early-stage colorectal cancer. Residual micrometastatic disease from the primary tumor is a major cause of patient relapse.

Experimental Design: To model colorectal cancer tumor cell invasion/metastasis, we have generated invasive (KRASMT/KRASWT/+ chr3/p53-null) colorectal cancer cell subpopulations. Receptor tyrosine kinase (RTK) screens were used to identify novel proteins that underpin the migratory/invasive phenotype. Migration/invasion was assessed using the XCELLigence system. Tumors from patients with early-stage colorectal cancer (N = 336) were examined for AXL expression.

Results: Invasive colorectal cancer cell subpopulations showed a transition from an epithelial-to-mesenchymal like phenotype with significant increases in migration, invasion, colony-forming ability, and an attenuation of EGF receptor (EGFR)/HER2 autocrine signaling. RTK arrays showed significant increases in AXL levels in all invasive sublines. Importantly, 5-FU treatment resulted in significantly increased migration and invasion, and targeting AXL using pharmacologic inhibition or RNA interference (RNAi) approaches suppressed basal and 5-FU–induced migration and invasion. Significantly, high AXL mRNA and protein expression were found to be associated with poor overall survival in early-stage colorectal cancer tissues.

Conclusions: We have identified AXL as a poor prognostic marker and important mediator of cell migration/invasiveness in colorectal cancer. These findings provide support for the further investigation of AXL as a novel prognostic biomarker and therapeutic target in colorectal cancer, in particular in the adjuvant disease in which EGFR/VEGF–targeted therapies have failed.

Introduction

Approximately 65% of all patients with colorectal cancer are initially diagnosed with early-stage (stage II/III) colorectal cancer. 5-fluorouracil (5-FU) or 5-FU/oxaliplatin chemotherapy following surgery is the current standard-of-care for patients with high-risk stage II and stage III colorectal cancer, respectively (1); however a large proportion of these patients will not derive any benefit from these treatments. The goals of adjuvant treatment are to eradicate residual local disease not removed by surgery and circulating micrometastatic disease. Epithelial–mesenchymal transition (EMT) has been suggested to play an important role in the initial invasion step during cancer metastasis (2). The success of standard adjuvant chemotherapy treatment may be limited as chemotherapeutic drugs preferentially kill proliferating cells, whereas residual micrometastatic disease may be nonproliferative or dormant (3).

Recent data from adjuvant phase III trials using the anti-VEGF monoclonal antibody (mAb) bevacizumab, or the EGF receptor (EGFR) mAb cetuximab have shown that, in contrast with the metastatic setting (4, 5), these agents do not prolong disease-free survival (DFS) in stage II/III colorectal cancer (6, 7). These results suggest that clinical advances and drug development in the metastatic setting cannot necessarily be directly translated to early-stage disease and suggest...
Translational Relevance

Despite the use of 5-fluorouracil (5-FU)-based adjuvant therapies, a large proportion of patients with high-risk stage II/III (early-stage/locally advanced) colorectal cancer will relapse and die of metastatic disease. Therefore, novel drugs are needed that result in further increase in overall survival (OS) in patients with early-stage colorectal cancer. In this study, we have generated invasive/migratory colorectal cancer cell subpopulations with epithelial-to-mesenchymal like phenotype, which showed significant increases in AXL expression levels. We further show that 5-FU, the cornerstone of adjuvant treatment, results in significant increased migration and invasion, and targeting AXL, using RNA interference (RNAi) and small-molecule inhibitor approaches, abrogated basal and 5-FU-induced migration/invasion. Importantly, high AXL expression levels were found to be associated with poor OS in early-stage colorectal cancer tissues. Thus, these results provide evidence to support the further evaluation of AXL as a prognostic biomarker and novel therapeutic target in colorectal cancer.

that primary tumors and metastatic lesions may represent distinct diseases driven by different tumor biology.

A number of studies have been focusing on the identification of the promoters and suppressors of invasion/metastasis and therapeutic modulation of these pathways. A recent study reported the acquisition of autocrine fibroblast growth factor receptor (FGFR)- or platelet-derived growth factor receptor (PDGFR)-signaling in non–small cell lung cancer (NSCLC) cells that have undergone an EMT transition, supporting the development of FGFR- or PDGFR-targeted therapies to reduce NSCLC tumor metastasis and progression (8). Other studies have identified salinomycin and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) as potential novel therapies for eradicating EMT-like/cancer stem cell-like cells (9, 10); however, none of these studies have yet been translated into the clinical setting.

AXL is a member of the TAM (Tyro3, AXL, and MER) receptor tyrosine kinase (RTK) family and was originally isolated as a transforming gene in cells from patients with chronic myeloproliferative disorders (11, 12). This subfamily is characterized by an extracellular domain, consisting of two immunoglobulin-like domains and two fibronectin type III (FNIII) motifs, a single transmembrane domain and an intracellular tyrosine kinase domain (13). AXL and MER share the vitamin K-dependent ligand growth-arrest-specific 6 (GAS6). Binding of GAS6 to AXL results in receptor dimerization, autophosphorylation of the tyrosine residues 779, 821, and 866, and recruitment of adaptor molecules. In other cases, ligand-independent dimerization and activation can also occur (14). AXL is ubiquitously expressed and detected in a wide variety of cells such as macrophages, platelets, and endothelial cells (15). Subsequent to its identification in chronic myelogenous leukemia, overexpression of AXL has been reported in a wide variety of cancers, such as breast, lung, and brain tumors (16–18).

Herein, we have generated progressively more invasive/metastatic cell populations from the KRASMT HCT116, KRASWT HCT116 (HKH-2), HCT116+Chr3, and HCT116-p53-null colorectal cancer cell lines, which exhibited morphologic, phenotypic, and molecular characteristics consistent with EMT. Using a RTK array, RNA interference (RNAi), and small-molecule inhibitor studies, we have identified AXL as a major regulator of migration/invasion and EMT. In addition, AXL expression was found to be a prognostic biomarker of poor overall survival (OS) in early-stage colorectal cancer tissues. Taken together, AXL-targeted therapies may represent a promising novel approach to prevent colorectal cancer recurrence and disease progression in early-stage colorectal cancer.

Materials and Methods

Materials
AZ/13032202 was obtained from AstraZeneca, R428 (BGB324; ref. 19) from BergenBio, and GAS6 from R&D systems. siRNAs targeting AXL (AXL_9, _10, _12, and _13), were purchased from Qiagen.

Cell culture
Authentication and culture of HCT116, HKH-2, and LoVo colorectal cancer cells have previously been described (20, 21). DLD-1 cells, provided by Senji Shirasawa (Fukuoka University, Fukuoka, Japan) in August 2008, were maintained in Dulbecco’s Modified Eagle Medium (DMEM) and authenticated by single-nucleotide polymorphism (SNP) profiling in January 2011. The LoVo (2004) cells were obtained from the European Collection of Cell Cultures. HCT116-p53-null and HCT116+chr 3 cells were provided by B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore) and CR. Boland (University of Michigan Medical School, Ann Arbor), respectively. COLO205 cells (2012) were obtained from the American Type Culture Collection [Authentication by short tandem repeat (STR) profiling/karyotyping/isoenzyme analysis] and maintained in RPMI medium.

Selection of invasive cells using Boyden chambers
BD BioCoat Matrigel Invasion Chambers (MGC; BD Biosciences) were used to isolate invasive subpopulations from colorectal cancer cells. Ten percent DMEM was added in the lower chamber. Colorectal cancer cell suspension in serum-free DMEM was seeded into the upper chamber.

Western blotting
Western blot analysis has previously been described (21, 22). Anti-E-cadherin (BD transduction laboratories), anti-SNAIL (Cell Signaling Technology), anti-STAT3 (Cell Signaling Technology), and anti-CD44 (Innitrogen) mouse monoclonal antibodies were used in conjunction with a
horseradish peroxidase (HRP)–conjugated sheep anti-mouse secondary antibody. Anti-AXL (Cell Signaling Technology) and anti-p-STAT3 (Y705; Cell signaling Technology) rabbit polyclonal antibodies were used in conjunction with an HRP-conjugated anti-rabbit secondary antibody.

**Immunofluorescence**

Of note, 3 × 10^5 cells were seeded overnight onto sterile coverslips. Cells were washed for 3 × 5 minutes in PBS and fixed in ice-cold 50:50 methanol (MeOH)/acetone. Cells were permeabilized and blocked in AB buffer (0.1% triton X-100, 5% goat serum, and 0.2% milk in PBS) for 1 hour followed by incubation in primary antibody (E-cadherin BD; 1:1,000) for 1 hour. Slides were then washed in AB buffer three times and incubated with goat anti-mouse Alexa Fluor 488 (Invitrogen Molecular Probes; 1:1,000) for 1 hour at room temperature, followed by washing as before. Counterstaining was with 4',6-diamidino-2-phenylindole (DAPI) DNA stain (Sigma-Aldrich; 0.1 μg/mL) before mounting in Vectashield.

**Real-time reverse transcription PCR analysis**

Total RNA and reverse transcription was carried out as previously described (23).

**Cell viability and clonogenic survival assays**

Cell viability assays were done as previously described (22, 23). Representative results of at least three independent experiments are shown.

**ELISA**

TGF-α, amphiregulin, and GAS6 ELISA assays were carried out as previously described (21).

**siRNA transfections**

siRNA transfections were done as previously described (20).

**In vitro migration and invasion assays**

Cell migration and invasion rates were assessed using the CIM-plate 16 and the XCELLigence system (Roche Applied Sciences) according to the manufacturer’s instructions.

**Generation of inducible AXL-silenced colorectal cancer cell lines**

HCT116 cells were transfected with 1 μg of pTRIPZ plasmid encoding Tet-inducible short hairpin RNA (shRNA) against AXL (Open Biosystems) using GeneJuice Transfection Reagent (Novagen). Stably transfected cells were selected, maintained in medium supplemented with 0.5 μg/mL puromycin (Life Technologies, Inc.) and induced with 2 μg/mL doxycycline (Sigma-Aldrich).

**Analysis of publicly available colorectal cancer datasets**

To assess the statistical association between AXL gene expression and clinical outcome, a publicly available colorectal cancer microarray dataset (NCBI GEO database; accession GSE17536; ref. 24) was accessed. The GSE17536 cohort consists of 177 patients with OS, DFS, and disease-specific survival (DSS) times and event/censoring status. Patients had a median age of 65.5 ± 13.1 years (25). There were 54.2% and 45.8% males and females, respectively. Of note, 13.6% (N = 24), 32.2% (N = 57), 32.2% (N = 57), and 22% (N = 39) had stage I, stage II, stage III, and stage IV colorectal cancer tumors respectively, and 9.0% (N = 16), 75.7% (N = 134), and 15.3% (N = 27) had well, moderately, and poorly differentiated tumors respectively. Normalized/log-transformed expression intensities for the AXL gene (probeset 202686_s_at) were extracted. Median OS was 134.86 months (73 events), DSS, 134.86 months (55 events), and DFS, undefined (36 events, 32 patients with no information). The expression data were in normalized, log-transformed format. tertile ranges for AXL expression values were identified. All patient samples were then allocated to one of three categories: AXL-LOW, AXL-MEDI-UM, and AXL-HIGH. Survival curves, comparing AXL-LOW (black) with AXL-HIGH (gray) expression groups for stage II/III were estimated with the Kaplan–Meier method and compared by the log-rank test, using GraphPad Prism version 5 for Windows, GraphPad Software.

**Clinicopathologic data from the patients for tissue microarray**

The study cohort consisted of 509 colorectal cancer cases that received resection of the primary tumor at the National University Hospital of Singapore between 1990 and 1999 (26). The available clinical and pathologic details are displayed in Supplementary Table S1. There were 7% (N = 37), 66% (N = 337), and 27% (N = 135) patients with stage I, stage II/III, and stage IV disease. This work was approved by the ethics committee of the National University of Singapore (NUS-IRB 131–05-017). The construction of the tissue microarray (TMA) was previously described (26). Methods of immunohistochemistry (IHC) have previously been described for CD133 (26), p53 (26), Ki67 (26), CD44 (27), and LGR5 (28). KRAS (codon 12/13) and BRAF (codon 600) mutational status was carried out as previously described (29, 30). In this study, we used anti-AXL antibody (Rabbit polyclonal; Sigma; 1:1,000). Staining intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). Analysis of staining in the normal colonic epithelium showed predominant absence or mild staining. In the colorectal cancer samples, grade 0 to 1 stain was classified as low expression, and grade 2 to 3 as high expression. Scoring was done independently by Tingting Wang and Supriya Srivastava.

**Statistical analysis**

All statistical analyses were performed using the SPSS package (version 15.0 for Windows, SPSS Inc.) with significance set at the 5% level. To test for correlations between the clinical features and the incidence of a respective mutation or immunohistochemical expression, we used the Kendall τ-b test for nonparametric parameters and Fisher exact test. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. The unpaired two-
tained t test was used to determine statistically significant differences between treatment effects. Significance was defined as $P < 0.05$. For univariate/multivariate analysis of Singapore data set, see Supplementary Methods.

**Results**

**Selection and characterization of invasive colorectal cancer subpopulations**

To identify targets that control invasion and metastasis in colorectal cancer, we generated a preclinical invasive colorectal cancer model using Matrigel Invasion Chambers (MIC). After 72- to 96-hour incubation, the cells that invaded through to the bottom chamber were collected and designated as “Invasive 1” (I1). These cells were propagated and repeatedly passed through the MIC until highly invasive HCT116-I6 cells were selected (Supplementary Fig. S1).

Using the xCELLigence system, we found significant increases in migration and invasion rates in the invasive HCT116 sublines, with a 4-fold and 20-fold increase in migration and invasion rate of HCT116-I6 cells compared with the parental HCT116 cells, respectively (Fig. 1A and B). Importantly, the increased migratory/invasion capacity seen in HCT116-I6 cells was a stable event and was not due to increased proliferation rates (Supplementary Fig. S2A and S2B).

Notably, in contrast with the parental HCT116 cells, which displayed an epithelial morphology, we found that the majority of invasive HCT116 (I3-I6) cells had transitioned to a spindle-shaped mesenchymal-like morphology (Fig. 1C and S2C). In keeping with the morphologic changes of EMT, we observed a loss of the epithelial marker E-cadherin and gain of the mesenchymal marker SNAIL in HCT116-I6 cells compared with the parental HCT116 cell line (Fig. 1D). Interestingly, we found that the expression of CD44, a marker associated with colorectal cancer stem cells (31), was approximately 4-fold higher in invasive HCT116-I6 cells than in control cells (Fig. 1D). To assess the tumor-initiating abilities of the selected invasive sublines, we carried out colony-forming assays and found an approximately 2-fold increased colony number in HCT116-I6 cell line compared with the parental HCT116 cells (Fig. 1E).

**The AXL RTK is overexpressed in colorectal cancer cells with increased migratory and invasive potential**

To identify the signaling mechanisms that drive the EMT phenotype in our colorectal cancer models, we assessed the phosphorylation status of 42 RTKs and found increased phosphorylation levels of the RTK AXL in the invasive HCT116-I3 cells compared with its parental cell line (Supplementary Fig. S2D). Validation of our array results by coimmunoprecipitation and Western blotting showed that constitutive phosphorylation of AXL was significantly increased in the invasive HCT116-I6 cells compared with its parental cell line, and this was associated with increased AXL protein and mRNA transcript levels (Fig. 2A and B).

We next assessed activation of EGFR family and insulin—like growth factor (IGF)-IR and also measured release of the prototypic EGFR ligands TGF-α and amphiregulin in the culture medium of parental and invasive HCT116 cells. Interestingly, TGF-α and amphiregulin levels and basal activity of EGFR, HER2, and IGF-IR were markedly decreased in the invasive HCT116 I1-I6 sublines (Fig. 2B and Supplementary Fig. S2E), suggesting that a switch...
away from epithelial RTK-signaling is an early event in the acquisition of an invasive, mesenchymal phenotype in colorectal cancer and that this is accompanied by increased expression of AXL. Notably, expression of GAS6 was undetectable in parental and invasive HCT116 cells (Supplementary Fig. S2F).

To rule out genotype-specific effects, invasive daughter lines were generated from KRAS WT-HCT116 (HKH-2; ref. 32), hMLH1-reconstituted MMR-proficient HCT116 (HCT116 + Chr3; ref. 33) and isogenic p53-null (HCT116-p53-null; ref. 34) cells (Supplementary Fig. S1). Similar to the data obtained in the KrasMT, p53WT, MMR-deficient HCT116 cell line, we found significant increases in the migration rates of invasive HKH-2, HCT116+Chr3, and HCT116-p53-null sublines compared with their parental cells, and this was associated with marked increased AXL and CD44 expression and decreases in EGFR and HER2 activity (Fig. 2C).

**AXL regulates colorectal cancer cell migration and invasion**

To further investigate the involvement of AXL in colorectal cancer cell migration and invasion, we used the small-molecule AXL inhibitors AZ13032202 and R428. AZ13032202 and R428 inhibited AXL tyrosine phosphorylation in both HCT116 parental and invasive I6 cells (Fig. 3A) and resulted...
in potent decreases in STAT3, Src family kinase (SFK), and Akt activity, basally and in response to the AXL ligand GAS6 (Fig. 3A and S3A). Treatment with AZ13032202 also resulted in potent decreases in migration rates of parental HCT116, LoVo, DLD-1, CACO-2, and COLO205 cell lines, but also of the invasive HCT116 cells (Fig. 3B and Supplementary Fig. S3C).

To exclude any off-target effects from AZ13032202 that may affect cell migration, we analyzed the effect of AXL-targeting siRNAs on migration. Silencing of AXL significantly reduced basal migration rates in parental and all invasive HCT116 cells (Fig. 3C and Supplementary Fig. S4A), and these effects were not due to increased cell death or decreased cell proliferation following AXL silencing (Supplementary Fig. S4B and S4C). Similar data were obtained in parental and invasive HKH-2 cells, DLD-1, and LoVo colorectal cancer cell lines (Fig. 3D). We also established

Figure 3. Pharmacologic inhibition and silencing of AXL reduces migration of parental and invasive colorectal cancer cells. A, lysates from HCT116 parental and invasive (I6) cells, treated with vehicle, AZ13032202, or R428 were immunoprecipitated (IP) with anti-phospho-tyrosine antibody and then immunoblotted (WB) for AXL. Protein expression of AXL, pERK1/2, ERK1/2, pSTAT3, STAT3, pSFK, Src, pAkt, and Akt were also analyzed. B, migration rates of parental and invasive HCT116 cells, LoVo, and DLD-1 colorectal cancer cells in absence and presence of AZ13032202. C, HCT116 parental and invasive sublines (I1-I6) were transfected with 5 nmol/L AXL siRNA for 24 hours and the effect on migration was determined using the XCELLigence system. D, migration of HCT116 parental and invasive (I6), HKH2 parental and invasive (I6), DLD-1, and LoVo cells, transfected with 5 nmol/L SC siRNA or 5 nmol/L AXL siRNA for 24 hours. E, migration of HCT116 cells stably transfected with the AXL lentiviral pTRIPZ vector system in absence and presence of doxycycline for 72 hours. shAxl 5.6, 5.7, 5.8 denote different clones. F, cell counts in HCT116 cells stably transfected with the AXL lentiviral pTRIPZ vector 24, 48, and 72 hours following incubation with vehicle or 2 μg/mL doxycycline. ** = P < 0.01; *** = P < 0.001.
doxycycline-inducible AXL shRNA clones (Fig. 3E), and assessed the effect of AXL targeting on proliferation and migration. Treatment of three individual AXL shRNA clones with doxycycline for 72 hours resulted in potent decreases in migration rates, similar to those observed with AZ13032202 and siRNAs (Fig. 3E and S4D), and this effect was not due to decreased cell proliferation in these clones (Fig. 3F). Collectively, all these data provide strong evidence that AXL is an important mediator of migration and invasion in colorectal cancer.

AXL regulates chemotherapy-induced migration and invasion

5-FU is the cornerstone of adjuvant treatment strategies for patients with early-stage colorectal cancer. In addition, our group has previously shown that exposure to 5-FU can activate a number of prosurvival pathways in colorectal cancer tumors (20, 21). In view of this, we examined the effect of 5-FU treatment on colorectal cancer cell migration and invasion and found dose-dependent, significant increases in both migration and invasion in the parental HCT116 cells (Fig. 4A and Supplementary Fig. S5A and S5B). These effects were not due to increased cell proliferation following 5-FU treatment (Supplementary Fig. S5B). Furthermore, a significant increase in invasion following 5-FU treatment was also observed in the invasive HCT116-I6 cells, but the migration rate was not further enhanced in this already highly migratory cell line (Fig. 4A and Supplementary Fig. S5A and S5C). 5-FU treatment also resulted in significant increases in migration of LoVo, COLO205, CACO2, and HCT116-chr3 cells, indicating that the effect of 5-FU on cell migration is not cell-line specific and not dependent on the microsatellite instability status (Fig. 4B and Supplementary Fig. S5D). Treatment with oxaliplatin resulted in further increase in migration when added to 5-FU (5-FU/oxaliplatin) in HCT116 cells (Supplementary Fig. S5E). Importantly, we found that the increased invasion and/or migratory potential following 5-FU treatment was abrogated following AXL gene silencing in parental and invasive HCT116 cells (Fig. 4A). Silencing of AXL also attenuated 5-FU-induced cell migration in LoVo and COLO205 cells (Fig. 4B). Furthermore, treatment of two individual AXL shRNA clones with doxycycline completely abrogated the increased migratory and invasion potential following 5-FU treatment (Fig. 4C and D). Western blot analyses demonstrated acute increased activity of STAT3, SFK, and Akt following treatment with 5-FU, and this was completely inhibited upon cotreatment with AZ13032202 (Fig. 4E). Importantly, AZ13032202 treatment also abrogated the increased migration following 5-FU treatment in HCT116 colorectal cancer cells (Fig. 4E). These results highlight a central role for AXL in regulating both an intrinsic and 5-FU-induced invasive/migratory phenotype in colorectal cancer.

AXL expression is a negative prognostic factor for survival in patients with early-stage colorectal cancer

To assess the clinical relevance of AXL expression in colorectal cancer, we initially accessed a publicly available colorectal cancer microarray dataset (24). Importantly, analysis of AXL mRNA expression (LOW-AXL vs. HIGH-AXL) in early-stage (stage II/III) colorectal cancer within this data set revealed a significant association between high AXL expression and decreased DSS ($P = 0.0265; N = 76$), DFS ($P = 0.0336; n = 74$), and OS ($P = 0.0497; n = 76$; Supplementary Fig. S6A). The multivariate analysis for AXL mRNA expression was not significant in this data set (Supplementary Table S2).

We also investigated AXL expression in 509 (stage I–IV) colorectal cancer samples by IHC (ref. 26; Supplementary Table S1). AXL was found to be highly expressed in colorectal cancer adenocarcinoma compared with normal colon tissue (Fig. 5A and Supplementary Fig. S6B). There were no significant correlations with KRAS mutational status ($P = 1.000$) or BRAF mutational status ($P = 0.546$) and AXL expression levels, supporting our preclinical findings (Supplementary Fig. S6C). There were also no significant associations with p53, Lgr5, CD133, or tumor cell proliferation by KI67 staining (Table 1). However, in support of our preclinical invasive models, strong correlation was found between AXL expression and CD44 staining ($P = 0.0005$; Table 1).

Next, we assessed the prognostic value of AXL expression in the 336 early-stage II/III colorectal cancer samples (subgroup of 509 patients with colorectal cancer) with mature survival data. The HR for OS for all stage II and III patients with high AXL expression was 1.406 [95% confidence interval (CI), 1.141–3.401], and showed a trend to statistical significance ($P = 0.0745$; Table 1). However, when the stage II/III was broken down into individual stages, there was a significant correlation between high AXL expression and poor 5-year OS when the stage II individuals were considered alone (HR, 1.970; 95% CI, 1.141–3.401; $P = 0.0149$; Fig. 5B). In both univariate and multivariate analyses of stage II patients, the level of AXL expression was the only significant factor with higher expression correlating with poorer prognosis (Table 2 and Supplementary Tables S3 and S4). AXL expression levels were retained in the final multivariate model with a concordance index of 0.571 (Table 2). Taken together, these data indicate that AXL expression is a predictor of poor clinical outcome in early-stage (stage II) colorectal cancer.

Discussion

Standard adjuvant therapies for patients with stage III and high-risk stage II colorectal cancer consists of 5-FU/ folinic acid/oxaliplatin, which also forms the control arms for most adjuvant trials. Recent phase III clinical trials failed to show any benefit from the addition of the biologic agents bevacizumab or cetuximab to standard 5-FU/FA/oxaliplatin chemotherapy treatment in early-stage colorectal cancer (6, 7). There is an urgent need to identify novel treatment strategies for early-stage colorectal cancer. In this study, we developed highly migratory/invasive colorectal cancer cell line models to identify potential novel targets/pathways driving colon cancer recurrence and metastasis. These invasive models displayed an EMT-
like morphology and phenotype, high levels of the stem-cell marker CD44 and increased colony-forming ability. Collectively, these data indicated that this in vitro invasive colorectal cancer model could provide new insights into understanding the biology of colorectal cancer cell invasion and metastasis.

Using a RTK array, AXL was identified to be highly activated in the invasive EMT-like colorectal cancer models. Furthermore, the increase in AXL activity in invasive/migratory colorectal cancer models was due to increase in AXL mRNA and protein expression. Consistent with a recent study in H358/doxZeb1, H358/doxSNAIL, and H358/TGFβ

Figure 4. AXL regulates basal and 5-FU–induced migration and invasion in colorectal cancer cells. A, HCT116 parental and invasive (I6) sublines were transfected with 5 nmol/L SC siRNA or 5 nmol/L AXL siRNA for 24 hours and migration and/or invasion rate was determined in absence and presence of 2 μmol/L 5-FU using the xCELLigence System. B, migration of LoVo and COLO205 cells transfected with 5 nmol/L SC siRNA or 5 nmol/L AXL siRNA in absence and presence of 2 μmol/L 5-FU. C, migration of HCT116 cells stably transfected with the AXL lentiviral pTRIPZ vector system in absence and presence of doxycycline for 72 hours and treated with 2 μmol/L 5-FU. D, invasion of HCT116 cells stably transfected with the AXL lentiviral pTRIPZ vector system in absence and presence of doxycycline for 72 hours and treated with 2 μmol/L 5-FU. E, left, expression of AXL, pERK1/2, ERK1/2, pSTAT3Y705, STAT3, pSFK, Src, pAkt, and Akt in HCT116 treated with AZ13032202, 5-FU, or cotreated AZ13032202 and 5-FU for 3 hours. Right, migration rate of HCT116 cells treated with vehicle or AZ13032202 in the absence or presence of 2 μmol/L 5-FU.
inducible "metastable" NSCLC cells (35), we found a shift from EGFR/HER2–activated autocrine signaling network to AXL signaling in KRASMT, KRASWT, +Chr3, and p53-null HCT116 cells, indicating that these effects are not dependent on the KRAS or p53 mutational or MSI status. In contrast with recent studies in NSCLC, we found no evidence of decreased sensitivity to EGFR inhibitors in the invasive KRAS WT, AXL overexpressing HKH-2 subline (Supplementary Fig. S7A; ref. 36). High AXL expression has been reported in several tumors, including esophageal (37), oral squamous cell (38), breast (16), lung (17), and malignant glioma cancers (18) and has been identified as a poor prognostic marker in these tumors. We demonstrate here for the first time, a key role for AXL as a regulator of migration and invasion in colorectal cancer.

Using RNAi and shRNA against AXL and several AXL small-molecule inhibitors, we further demonstrated potent inhibition of migration and invasion in a panel of parental and invasive colorectal cancer cell lines. In contrast with a previous study (9), we found no increased resistance to chemotherapy treatment, in particular 5-FU, in the EMF/stem-cell like HCT116 invasive cells (Supplementary Fig. S7B). However, this is the first study showing that 5-FU treatment, the cornerstone of adjuvant treatment in colorectal cancer, significantly increases both the migratory and invasive potential of colorectal cancer cells and invasion of invasive colorectal cancer sublines. Importantly, we found that sh/siRNA against AXL and the small-molecule inhibitor AZ13032202 abrogated the increased migratory and/or invasion potential following 5-FU treatment in parental and invasive colorectal cancer cells. Taken together, these studies would indicate that inhibitors of the AXL pathway alone or in conjunction with 5-FU chemotherapy could be a novel treatment strategy for early-stage colorectal cancer. Nevertheless, other studies investigating the molecular changes associated with EMT transition have identified increased autocrine IL6-JAK2-STAT3 and PDGFR/FGFR signaling together with increased AXL/Tyro3 signaling, suggesting that pharmacologic inhibition of this survival network may result in reduced cancer progression and recurrence (35).

A number of studies have shown that AXL can signal through the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and NF-κB pathways, resulting in increased cell proliferation, survival (39), tumor growth (40), and resistance to chemotherapy (41). Consistent with previous studies in breast cancer, we found strong inhibition of basal and 5-FU–induced Akt activity following AXL inhibition (19). In addition, 5-FU treatment also resulted in increases in SFK and STAT3 activity and both basal and 5-FU–induced upregulation of pSTAT3 and SFK were potently abrogated following AXL inhibition. Several
studies have shown key roles for both SFK and STAT3 signaling pathways in the growth of stem-cell like cancer cells (42, 43) and cancer cell migration (44, 45). In addition, a recent study showed that high STAT3 activity is positively associated with peritumoral lymphocytic reaction and shorter OS in colorectal cancer, suggesting its potential role as a therapeutic target in colorectal cancer (46). Taken together, these studies would suggest that

### Table 1. AXL is an independent prognostic biomarker

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<td>Ki67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>108</td>
<td>209</td>
<td>–0.035</td>
<td>0.445</td>
</tr>
<tr>
<td>High expression</td>
<td>72</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD133</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>99</td>
<td>208</td>
<td>–0.080</td>
<td>0.072</td>
</tr>
<tr>
<td>High expression</td>
<td>81</td>
<td>121</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** To test correlation between p53, CD44, LGR5, Ki67, CD133, and AXL expression in colorectal cancer TMA, we used the Kendall $t$-$b$ test for nonparametric parameters and Fisher exact test. * = significant.

### Table 2. Univariate and multivariate analyses in stage II colorectal cancer group using Cox proportional hazards regression

<table>
<thead>
<tr>
<th>Stage II</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>CI</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.9787</td>
</tr>
<tr>
<td>Ethnic group</td>
<td>Chinese</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Non-Chinese</td>
<td>0.7749</td>
</tr>
<tr>
<td>Tumor size (mm; linear term)</td>
<td>Unit increase</td>
<td>1.0423</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0.8992</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.065</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.002</td>
</tr>
<tr>
<td>AXL</td>
<td>Low</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2.217</td>
</tr>
</tbody>
</table>

**NOTE:** Concordance index, 0.571.
activation of the SFK and/or STAT3 signaling pathways following 5-FU treatment leads to enhanced migratory/invasive potential, which can be reversed by AXL inhibition.

To investigate the clinical importance of AXL in colorectal cancer, we measured AXL expression in colorectal cancer tissues and found high levels of AXL in primary colorectal cancer tumors compared with normal tissue. Further investigation of AXL mRNA expression in public available data sets revealed that high AXL mRNA expression was significantly associated with poorer DFS, DSS, and OS in patients with stage II/III colorectal cancer. Importantly, further univariate and multivariate analyses of our colorectal cancer TMA revealed also that stage II colorectal cancer patients with high AXL expression levels have a shorter 5-year OS compared with stage II patients with low AXL expression. We now plan a further retrospective validation of the prognostic role of AXL in a large cohort of stage II colon cancer samples as part of a clinical trial. The clinical management of patients with stage II colorectal cancer is a topic of debate with 5-year OS rates between 80% and 85% (47). Currently, only stage II patients with poor prognostic clinical–pathologic features such as lymphovascular invasion, peritoneal involvement, poor differentiation, obstruction, and perforation will receive adjuvant 5-FU treatment. There is an urgent need to identify approximately 15% to 20% of patients who are most likely to relapse and will benefit from adjuvant treatments. A number of studies, including our own prognostic signature study (48, 49), have attempted to develop prognostic biomarkers in early-stage colorectal cancer. The results herein obtained from both public available data sets and colorectal cancer TMA suggest that AXL may be a biomarker of poor prognosis in stage II colorectal cancer.

In conclusion, we have generated highly migratory/invasive colorectal cancer cell line models with EMT/stem-cell like phenotype. RTK screen identified AXL as a key regulator of colorectal cancer cell migration and invasion. Further functional validation showed that AXL regulates colorectal cancer migration and invasion in colorectal cancer cell line models, as single agent and in the context of 5-FU treatment. Importantly, AXL is highly expressed in colorectal cancer and is a poor prognostic biomarker in stage II colorectal cancer disease. The data suggest that inhibition of AXL alone or in combination with 5-FU treatment may represent a promising treatment strategy in colorectal cancer, in particular in adenant disease in which EGFR-and VEGF-targeted therapies have failed.

Disclosure of Potential Conflicts of Interest

P.C. Johnston is director of Almac Diagnostics and has an ownership interest (including patents) in both Almac Diagnostics and Fusion Antibodies. He is a consultant/advisory board member of Chugai Pharmaceuticals, Sanofi-Aventis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: P.D. Dunne, D.G. McArt, J.K. Blayney, M. Kalimutho, S. Srivastava, K. Redmond, D.B. Longley, M. Salto-Tellez, P.G. Johnston, S. Van Schaeybroeck
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.D. Dunne, D.G. McArt, M. Loughrey, P.G. Johnston, S. Van Schaeybroeck
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