EphA2 Expression Is a Key Driver of Migration and Invasion and a Poor Prognostic Marker in Colorectal Cancer

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Abstract

Purpose: EphA2, a member of the Eph receptor tyrosine kinases family, is an important regulator of tumor initiation, neovascularization, and metastasis in a wide range of epithelial and mesenchymal cancers; however, its role in colorectal cancer recurrence and progression is unclear.

Experimental Design: EphA2 expression was determined by immunohistochemistry in stage II/III colorectal tumors (N = 338), and findings correlated with clinical outcome. The correlation between EphA2 expression and stem cell markers CD44 and Lgr5 was examined. The role of EphA2 in migration/invasion was assessed using a panel of KRAS wild-type (WT) and mutant (MT) parental and invasive colorectal cancer cell line models.

Results: Colorectal tumors displayed significantly higher expression levels of EphA2 compared with matched normal tissue, which positively correlated with high CD44 and Lgr5 expression levels. Moreover, high EphA2 mRNA and protein expression were found to be associated with poor overall survival in stage II/III colorectal cancer tissues, in both univariate and multivariate analyses. Preclinically, we found that EphA2 was highly expressed in KRASMT colorectal cancer cells and that EphA2 levels are regulated by the KRAS-driven MAPK and RalGDS-RalA pathways. Moreover, EphA2 levels were elevated in several invasive daughter cell lines, and downregulation of EphA2 using RNAi or recombinant EFNA1 suppressed migration and invasion of KRASMT colorectal cancer cells.

Conclusions: These data show that EphA2 is a poor prognostic marker in stage II/III colorectal cancer, which may be due to its ability to promote cell migration and invasion, providing support for the further investigation of EphA2 as a novel prognostic biomarker and therapeutic target.

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Introduction

Despite improvements in overall survival (OS) with the introduction of adjuvant 5-FU/folinic acid/oxaliplatin treatment for patients with locally advanced stage II and III colorectal cancer, the management of these patients remains an area of active clinical debate. Although the molecular targeted agents bevacizumab, cetuximab, panitumumab, afiblercept, and ramucirumab have each improved outcome of colorectal cancer patients with metastatic disease, no benefit from these agents has been seen in stage II/III colorectal cancer (1, 2). Understanding the molecular mechanisms of disease progression and resistance to available treatments, with the subsequent development and validation of novel therapeutic strategies, is therefore urgently required in order to develop stratified medicine approaches that individualize patient treatment in stage II/III disease.

EphA2 is a 130 kDa glycoprotein receptor and belongs to the largest family of receptor tyrosine kinases (RTK), the Eph family. The Eph family can be divided into two subclasses, EphA and EphB, based on structural homology and affinity for binding either the GPI-anchored Ephrin-A or the transmembrane Ephrin-B ligands (3). Both EphA and EphB contain an extracellular region with an Ephrin binding domain, an epidermal growth factor-like motif, and two fibronectin type-III domains as well as a cytoplasmic region, including a juxtamembrane segment, the kinase domain, a sterile α-motif (SAM), and a PDZ-binding motif (postsynaptic density protein, Drosophila disc large tumor suppressor, and Zonula occludens-1 protein). Eph–Ephrin complexes emanate bidirectional signaling into both Eph-expressing cells (forward signaling) and Ephrin-expressing cells (reverse signaling; ref. 4). In addition to ligand-dependent receptor activation, some studies have shown that even without ligands, Eph...
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Materials and Methods

Materials

MG132 and cycloheximide were obtained from Sigma-Aldrich, Trametinib (GSK1120212) and Vemurafenib (PLX4032) from Selleck Chemicals LLC, and rhEFA1 from R&D systems. siRNAs targeting EphA2, EFNA1, KRAS, RalGDS, RalA, RalB, PLCe, TIAM1, and PIK3CA were purchased from Qiagen. siRNAs targeting AKT1, AKT2, AKT3, p53, p63, p21, c-Src, YES, and FYN were obtained from Dharmacon. The EphA2 plasmid was a kind gift from Dr. Koichi Miura (Osaka, Japan; ref. 12).

Cell culture

Authentication and culture of HCT116, HKH-2, DLD-1, Dks-8, LoVo, LS174T, SW620, GPSD, RKO, WiDr, HT-29, LIM2405, and Caco-2 colorectal cancer cells have previously been described (13–15). COLO205 and CDD-18Co cells (2012) were obtained from the American Type Culture Collection [authentication by short tandem repeat (STR) profiling/karyotyping/isonzyme analysis] and maintained in RPMI and EMEM, respectively. HCT116-p53 wild type, HCT116-p53 null (+/-), HCT116-p53 (++), HCT116-p53 (R248W/-), and HCT116-p53 (R248W+) cells were provided by B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore). The DiFi and OXCO-2 cells were received from Dr. Di Nicantonio in March 2015 (University of Turin, Italy).

Immunofluorescence

Immunofluorescence has previously been described (17). Anti-EphA2 (Invitrogen; 1:500) mouse monoclonal antibody was used in conjunction with a horseradish peroxidase (HRP)–conjugated sheep anti-mouse secondary antibody. Anti-pEphA2Y588, anti-pEphA2S597, anti-pEphA2Y772, anti-RalB, c-Src, YES, and FYN (Cell Signaling) rabbit polyclonal antibodies were used in conjunction with a HRP-conjugated anti-rabbit secondary antibody. Anti-EphA2 (Cell Signaling) and anti-pTyrosine (Cell Signaling) rabbit antibodies were used for coimmunoprecipitation.

Real-time reverse transcription PCR analysis

RNA was isolated using the GeneJET RNA purification Kit (Thermo Scientific) and reverse transcribed using the Moloney murine leukemia virus-based reverse transcriptase Kit (Invitrogen). Q-PCR analysis was performed using the LightCycler 480 probes master mix (LightCycler 480II; Roche).

siRNA transfections

siRNA transfections were performed as previously described (13).

Generation of inducible EphA2-silenced colorectal cancer cell lines

Inducible EphA2-silenced HCT116 cells were generated as previously described (17). A pTRIPZ plasmid encoding Tet-inducible shRNA against EphA2 was used (Open Biosystems).

Clinical–pathologic data

The study cohort consisted of 509 stage I–IV colorectal cancer cases who received resection of the primary tumor at the National
The final dataset for survival analysis consisted of 335 stage II/III patients. The available clinical and pathologic details, construction of the tissue microarray, and methods of immunohistochemistry (IHC) for CD133, Ki-67, CD44, LGR5, and AXL have previously been described and ethically approved for research (NUS-IRB 131–05-017). The TMA contained 1 core per colorectal tumor. In this study, we used anti-EphA2 antibody (Mouse monoclonal; Invitrogen; 1:100). Staining intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining); categories 0 and 1 were classified EphA2-low and categories 2 and 3 as EphA2-high. Scoring was done independently by Tingting Wang and Supriya Srivastava, who were both blinded to clinical outcome. In addition to EphA2 levels, OS and survival status (death by any cause was considered an event), age, gender, tumor size, ethnic group (Chinese/non-Chinese), invasion (either perineural and/or lymphatic and/or vascular), differentiation (1, 2, or 3), tumor site (rectal or colon), chemotherapy status, and staging were available for each patient. Patients with an event occurring less than 3 months after resection were excluded from the analysis, resulting in a revised stage II/III dataset of 313 patients (Supplementary Table S1A and S1B).

EphA2 expression in normal colonic epithelium and colorectal cancer was analyzed using a tissue microarray (TMA) consisting of cores representing colorectal adenocarcinoma with matched normal colon tissue from 211 stage II/III colorectal cancer patients (21). This work was approved by the Office for Research Ethics Committees Northern Ireland (08/NIR02/105).

Validation cohort
An independent validation dataset was identified, and the normalized, log-transformed data were obtained from the Gene Expression Omnibus (GEO) database, accession number GSE17536 (22, 23). The stage II/III patients were selected (n = 114) and the probe set corresponding to EphA2 identified (203499_at). The distribution with respect to EphA2 from the main study (stage II/III: low: 49%; high: 51%) was applied to the remaining patients, and a patient was therefore labeled as EphA2-low or EphA2-high. The factors age and stage were also extracted as were OS and survival status.

Statistical analysis
The unpaired two-tailed Student t test was used to determine statistically significant differences between treatment effects and calculated using GraphPad Prism version 5 for Windows (GraphPad Software). Significance was defined as P < 0.05. The non-parametric tau test was used to determine the Kendall rank correlation coefficient as a measure of association between EphA2 expression and other markers. Subsequently, the Fisher exact test and Bonferroni method (Statistical Package for the Social Sciences; SPSS) were used to determine the significance of the association. Methods on survival and univariate/multivariate
Figure 2.
KRAS and downstream effectors MEK1/2 and RalGDS regulate EphA2 expression. A, left, HCT116 cells were transfected with 10 nmol/L SC or 10 nmol/L EphA2, KRAS, BRAF, ERK1, ERK2, or ERK1/ERK2 siRNA for 24 hours and levels of EphA2, KRAS, BRAF, pERK1/2, ERK1/2, pAKT, and AKT determined (LE, long exposure; SE, short exposure). Middle, HCT116 cells were transfected with 10 nmol/L SC or 10 nmol/L KRAS siRNA for 24 hours, levels of EphA2 and EFNA1 were determined by real-time PCR. (Continued on the following page.)
analysis of Singapore dataset and validation cohort are further described in Supplementary Methods.

Results

Oncogenic KRAS is associated with high EphA2 expression levels in colorectal cancer cell lines

Previous KRAS siRNA screens from our lab have identified the RTK EphA2 as a potential KRAS target gene in KRASMT colorectal cancer cells (15). Based on these results, we analyzed EphA2 expression and phosphorylation levels in the KRASMT HCT116 and DLD-1 and KRASWT HKH-1 and Dks-8 isogenic paired cell line models using Western blotting and coimmunoprecipitation (24). Constitutive EphA2 levels were significantly higher in the KRASMT HCT116 and DLD-1 cells compared with their KRASWT counterparts (Fig. 1A; Supplementary Fig. S1A). Phosphorylation levels of EphA2 at Y772 (tyrosine residue within the activation loop), S897 (ligand-independent serine residue), and Y588 (tyrosine residue located in the juxtamembrane region which controls its kinase activity) were likewise higher in KRASMT cells compared with their isogenic WT clones, suggesting that EphA2 is actively signaling in the KRASMT models (25–27). We also found increased EphA2 mRNA levels in the HCT116 cells compared with its WT clone (Fig. 1B). Moreover, significantly higher mRNA levels of EFNA1 (a major ligand for EphA2) were found in the KRASMT HKH-2 cell line, consistent with previous data showing an inverse correlation between expression of EphA2 and EFNA1 in breast cancer cells (Fig. 1B; ref. 28). Silencing of EFNA1 markedly increased EphA2 expression levels in HCT116 and HKH-2 cells, indicating that EFNA1 negatively regulates EphA2 levels in colorectal cancer (Supplementary Fig. S1B). Furthermore, the half-life of EphA2 was markedly reduced in the HKH-2 cells, with no change and a 72% reduction in EphA2 levels 24 hours following treatment with the protein synthesis inhibitor cycloheximide in HCT116 and HKH-2 cells, respectively (Supplementary Fig. S1C). These data would suggest that in addition to differences in transcriptional regulation, the increased EphA2 levels in KRASMT HCT116 are a result of decreased protein turn-over and increased protein stability.

Recent studies in lung cancer and glioma have suggested a role for EphA2 in regulating cancer stem–like properties (29, 30). Interestingly, we found that expression of CD44, a marker associated with colorectal cancer stem cells (31), was higher in KRASMT cells and correlated with high expression levels of EphA2 (Fig. 1A; Supplementary Fig. S1D). We also found high expression and phosphorylation levels of EphA2 and CD44 in a panel of nonmatched KRASMT and BRAFMT colorectal cancer compared with KRASWT/BRAFVT colorectal cancer cells (Fig. 1C).

The RAS/MEK and RAS/RalGDS/RalA pathways regulate EphA2 expression levels

Given the differential expression of EphA2 in KRASMT, BRAFMT, and KRASWT colorectal cancer cells, we hypothesized that KRAS may directly affect EphA2 expression. siRNA against KRAS or treatment with the MEK1/2 inhibitor GSK1120212 resulted in strong decreases in EphA2 mRNA and protein levels and significant increases in EFNA1 mRNA levels in KRASMT HCT116 cells (Fig. 2A). Similar results were also obtained in a panel of KRASMT and BRAFMT colorectal cancer cells using GSK1120212 (Supplementary Fig. S2A). Interestingly, treatment with the BRAF inhibitor vemurafenib decreased EphA2 levels in BRAFMT cells, but not in KRASMT cells (Supplementary Fig. S2B); the sustained MAPK signaling following vemurafenib or siBRAF in the KRASMT colorectal cancer cells is consistent with previous studies showing that inhibition of BRAF in the presence of oncogenic RAS induces BRAF binding to CRAF, leading to CRAF hyperactivation and sustained MEK1/2-ERK1/2 activation (32).

Furthermore, RNAi against KRAS resulted in more potent inhibition of EphA2 expression compared with the effects of siERK1, siERK2, or combined siERK1/2, indicating that other KRAS effector pathways may regulate EphA2 expression (Fig. 2A). To further investigate the role of the additional effector pathways for RAS, we used RNAi against AKT1/2/3, PI3KCA, PLC, TIAM1, and RalGDS and found that EphA2 levels were potently decreased by RalGDS gene silencing, whereas silencing of the other major RAS effectors did not significantly affect EphA2 levels (Fig. 2B; Supplementary Fig. S2C). RalGDS is a Guanine nucleotide Exchange Factor (GEF), coupling RAS to the GTPases RaLa and RaLB (33). Silencing of RaLa, but not RaLB, resulted in significant decreases in EphA2 mRNA and protein levels in HCT116 and DLD-1 cells (Fig. 2C; data not shown). There was no effect of siRaLa on EFNA1 mRNA expression levels (Supplementary Fig. S2D). We also explored a potential regulation of EphA2 by p53 or the Src family kinases (SKF), using RNAi and the isogenic HCT116-p53wt, HCT116-p53 null (−/−), HCT116-p53 (+/−), HCT116-p53 (R248W/−), and HCT116-p53 (R248W+/+) cells. These data showed that EphA2 is not regulated by p53 or the SKFs (Supplementary Fig. S2E and S2F). Taken together, these results would indicate that KRAS regulates EphA2 expression through both the MAPK and RalGDS-RaLa pathways in KRASMT colorectal cancer models (Fig. 2D).

EphA2 regulates migration and invasion in colorectal cancer

Previous studies have shown a role for EphA2 in invasion and metastasis in breast, glioblastoma, and NSCLC (9, 25, 30). To investigate a potential role for EphA2 in invasion and migration in colorectal cancer, we initially assessed expression and phosphorylation levels of EphA2 in the KRASMT, KRASWT (HKH-1), and KRASMT/+Chr3 HCT116 parental and invasive sublines, previously generated in our lab using Matrigel Invasion Chambers (17). We found significant increases in EphA2 mRNA and protein expression and phosphorylation levels in the invasive HCT116, HKH-1, and HCT116+Chr3 sublines compared with their
### Figure A

**Clonal viability and migration assays**

<table>
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<th>Cell Line</th>
<th>Treatment</th>
<th>Migration Rate</th>
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<tr>
<td>HCT116</td>
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</tr>
<tr>
<td>HKH2</td>
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</tr>
<tr>
<td>HKH2</td>
<td>HKH2-6</td>
<td>0.20</td>
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**Relative migration rate**

- **Untreated**: SC
- **2 μg/mL Doxycycline**: SC

**Figure B**

**Invasion assays**

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<td>DLD-1</td>
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**Relative invasion rate**

- **EV**: p-cDNA 3.1-EphA2
- **EV + Doxycycline**: p-cDNA 3.1-EphA2

**Figure C**

**IP: p-Tyrosine**

<table>
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<th>Time</th>
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<td>Unt A1</td>
<td>Unt A1</td>
</tr>
<tr>
<td>30 min</td>
<td>Unt A1</td>
<td>Unt A1</td>
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**Figure D**

**Migration assays**

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<th>Migration Rate</th>
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<tr>
<td>HCT116</td>
<td>rhEFNA1 100 ng/mL</td>
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<td>DLD-1</td>
<td>PBS</td>
<td>0.50</td>
</tr>
<tr>
<td>DLD-1</td>
<td>rhEFNA1 100 ng/mL</td>
<td>0.15</td>
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</tbody>
</table>
parental cells, and this was associated with markedly increased CD44 expression levels (Fig. 3A; Supplementary Fig. S3A). In addition, we also found marked decreases in EFNAl mRNA levels in the HKH–2–invasive cells, compared with its parental cell line (Supplementary Fig. S3A). Silencing of EphA2 significantly reduced basal migration and invasion rates in KRASMT HCT116, DLD-1, and LoVo cells and in the invasive HCT116 and HKH–2 cells (Fig. 3A; Supplementary Fig. S3B; data not shown).

We also established doxycycline-inducible EphA2 shRNA clones and assessed the effect of EphA2 targeting on migration. Treatment of two individual EphA2 shRNA clones with doxycycline for 72 hours resulted in potent decreases in migration rates similar to those observed with siRNA (Fig. 3A). Furthermore, the CD44+ subpopulation was significantly reduced in the corresponding EphA2 knockdown cells (Supplementary Fig. S3C). Importantly, transient overexpression of exogenous EphA2 resulted in increased invasion and migration of colorectal cancer cells (Fig. 3B; Supplementary Fig. S3D).

Our group has previously shown that exposure to 5-FU significantly increases both migration and invasion of colorectal cancer cells (17). We now show that 5-FU treatment results in acute increases in EphA2 expression levels and that silencing of EphA2 abrogates the increased migratory potential following 5-FU treatment in colorectal cancer cells (Fig. 3B).

We also investigated the effect of the EphA2 ligand EFNAl on colorectal cancer cell migration. Incubation of HCT116 cells with rhEFNA1 increased EphA2 tyrosine phosphorylation and resulted in EphA2 internalization and decreased protein expression after 15 and 30 minutes, respectively (Fig. 3C, upper middle; Supplementary Fig. S3E). Immunoprecipitation experiments confirmed that EphA2 interacts with Cbl (an ubiquitin E3 ligase) in HCT116 following incubation with rhEFNA1 for 15 minutes (Fig. 3C, bottom). Moreover, pretreatment with the proteasome inhibitor MG132 attenuated rhEFNA1-induced EphA2 downregulation, providing further evidence that EphA2 downregulation is mediated by the ubiquitin proteasome system (Fig. 3C, bottom). Importantly, incubation of colorectal cancer cells with rhEFNA1 significantly reduced basal migration rates in HCT116, DLD-1, and LoVo KRASMT cells, similar to those observed with RNAi against EphA2 (Fig. 3D; Supplementary Fig. S3F).

In view of our previous data showing that RaLa regulates EphA2 expression levels, we also determined the effect of siRaLa on migration of KRASMT colorectal cancer cells. Silencing of RaLa significantly reduced basal migration rates in HCT116, DLD-1, and LoVo cells (Fig. 3D; Supplementary Fig. S3F). Collectively, these data provide strong evidence that EphA2 is an important mediator of migration and invasion in colorectal cancer and that downregulating EphA2 using RNAi or rhEFNA1 or by blocking upstream regulators of EphA2 expression, such as RaLa, abrogates migration of KRASMT colorectal cancer cells.

**EphA2 is differentially expressed in colorectal cancer versus matched normal tissue**

To investigate the clinical importance of EphA2 in colorectal cancer, we assessed EphA2 expression in 211 (stage II–III) colorectal cancer samples by IHC (21). EphA2 was found to be highly expressed in colorectal adenocarcinoma compared with matched normal colon tissue (Fig. 4A). In contrast with our preclinical models, no correlation between KRAS mutational status and EphA2 expression levels was found within this dataset (data not shown). High EphA2 expression levels were also found in a second TMA, including 509 stage I–IV colorectal cancer samples (Supplementary Fig. S4A; ref. 18). In support of our preclinical data, a strong correlation was found between EphA2 expression and the stem cell markers CD44 (P < 0.0001) and Lgr5 (P < 0.0001), both in the entire patient cohort and when the early stage II/III colorectal tumors were considered alone (Table 1; Supplementary Table S2). In both surgery-only and surgery/chemotherapy cohorts, no significant statistical associations between pairs of clinical pathologic factors were identified (Supplementary Table S3; data not shown).

**EphA2 expression is a negative prognostic factor for survival in early-stage colorectal cancer**

Next, we assessed the prognostic value of EphA2 expression in the 313 stage II/III colorectal cancer (subgroup of 509 colorectal cancer patients) samples with mature survival data (ref. 18; Supplementary Table S1A). In univariate analysis, there was a significant correlation between high EphA2 expression and poor OS (P = 0.0408; Fig. 4B). When the stage II/III group was broken down into surgery-only and surgery/adjuvant chemotherapy cohorts, increased age [HR, 1.06; 95% confidence interval (CI), 1.03–1.09; P = 0.00033], stage III (HR, 2.52; 95% CI, 1.51–4.19; P = 0.00388), and high levels of EphA2 (HR, 1.97; 95% CI, 1.20–3.25; P = 0.007890) were associated significantly with poorer prognosis when the surgery-only group was considered alone.

**Figure 3.**

EphA2 reduces migration in KRASMT CRC cells. A, left, EphA2, EphA2S897, EphA2Y722, and CD44 levels in parental (P) and invasive CRC sublines (I6). Upper right, migration of parental and invasive (I6) cells, transfected with 10 nmol/L SC or 10 nmol/L EphA2 siRNA. Lower right, migration of HCT116 cells stably transfected with the EphA2 lentiviral pTRIPZ vector system in the absence and presence of doxycycline for 24 hours. shEphA2 3.5 and 3.8 denote different clones. EphA2 expression was determined by WB. B, left, CRC cells were transiently transfected with EV (PCDNA 3.1) or EphA2 expression construct for 24 hours, and invasion rates determined using the xCELLigence system. EphA2 levels were determined by WB. Right, migration of HCT116 cells stably transfected with the EphA2 lentiviral pTRIPZ vector system in the absence and presence of doxycycline for 72 hours and treated with 5-FU. EphA2 expression was determined by WB. C, left upper, lysates from HCT116 cells treated with vehicle or rhEFNA1 were immunoprecipitated (IP) with anti-phospho-tyrosine antibody and then immunoblotted (WB) for CBL. Protein expression of EphA2 and CBL was also analyzed. Lower right, HCT116 cells were treated with 0.1 μmol/L MG132 for 1 hour, followed by stimulation with 100 ng/mL rhEFNA1 (A1) for 15 minutes. Lysates were immunoprecipitated with anti-EphA2 antibody and then immunoblotted (WB) for CBL. Protein expression of EphA2 and CBL was also analyzed. Lower right, HCT116 cells were treated with 0.1 μmol/L MG132 for 1 hour, followed by stimulation with 100 ng/mL rhEFNA1 (A1) for 1 hour and EphA2 expression levels determined. D, migration of colorectal cancer cells, incubated with 100 ng/mL rhEFNA1 for 24 hours or transfected with 10 nmol/L SC or 10 nmol/L RaLa siRNA. Error bars represent mean ± SD of quadruplicate values from one of 3 independent experiments.
Table 1. EphA2 and clinical-pathologic correlates in colorectal cancer. Correlation between EphA2 and CD44, LGR5, CD133, Ki-67, and AXL in 338 stage II/III colorectal cancer cases of the Singapore dataset.

<table>
<thead>
<tr>
<th>Stage II/III</th>
<th>Low expression (0–1) (n = 165)</th>
<th>High expression (2–3) (n = 173)</th>
<th>Kendall’s tau</th>
<th>Fisher exact P (Bonferroni adjusted)</th>
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<tr>
<td>Features</td>
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<td>Low expression</td>
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<tr>
<td>High expression</td>
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<td>LGR5</td>
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<td>High expression</td>
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<tr>
<td>High expression</td>
<td>51</td>
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<td>Ki-67 expression</td>
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(Fig. 4C, left; Table 2A). Subsequently, we developed a full multivariate model and found that increased age (HR, 1.06; 95% CI, 1.03–1.09; P = 0.00044), stage III (HR, 2.97; 95% CI, 1.66–5.32; P = 0.00025), and high levels of EphA2 (HR, 1.97; 95% CI, 1.09–3.56; P = 0.024) were correlated with an increased risk of death in the surgery-only group (Table 2A). The final multivariate model resulted in a concordance index (c-index) of 0.75. High EphA2 level, increased age, and stage III were all associated with poorer prognosis in the surgery-only group (Table 2B). In contrast, there was no significant association between EphA2 expression and prognosis in the resection/adjuvant chemotherapy cohort, suggesting a treatment interaction effect (Supplementary Fig. S4B and Supplementary Table S4). Further subanalysis for patients with stage II or stage III disease alone showed that high EphA2 expression was prognostic for poor OS in the stage II surgery-only cohort (P = 0.000472) and the stage III surgery-only cohort (P = 0.0375) but not in the stage II surgery-chemotherapy cohort (P = 0.434) or the stage III surgery-chemotherapy subgroup (P = 0.441; Supplementary Figs. S4C and S5).

Independent validation of EphA2 as a poor prognostic biomarker in colorectal cancer

The prognostic role of EphA2 expression in colorectal cancer was validated using a publicly available stage II/III (n = 114) colorectal cancer microarray dataset (GSE17536; refs. 17, 34). Analysis of EphA2 mRNA expression in this dataset revealed a significant association between high EphA2 expression and decreased OS (P = 0.0277; Fig. 4C, right). The multivariate model showed that stage III (HR, 2.52; 95% CI, 1.20–5.27; P = 0.014000) and high EphA2 expression (HR, 2.31; 95% CI, 1.15–4.67; P = 0.019) were associated with poorer OS (Table 2C). The model resulted in a c-index of 0.705. Taken together, these data indicate that EphA2 expression has the potential of predicting poor clinical outcome in early stage II/III colorectal cancer.

Growth factors regulate EphA2 levels in KRASWT colorectal cancer cells

Given the lack of correlation between EphA2 levels and KRAS status in our clinical samples, we determined whether the tumor microenvironment could influence EphA2 levels in KRASWT models. We found markedly increased EphA2 levels in the KRASWT HKH-2, DiF, OXCO2, and Dks-8 cells following incubation with recombinant human EGF, TGFα, or HGF, thus supporting the idea that microenvironment-derived ligands can regulate EphA2 levels in KRASWT colorectal cancer (Fig. 4D, left; Supplementary Fig. S6A). In addition, incubation of HKH-2 cells with conditioned medium from CCD-18Co colon fibroblasts, or coculture of HKH-2 cells with CCD-18Co cells, resulted in potent increases in EphA2 expression levels in the HKH-2 cell line (Fig. 4D, right; Supplementary Fig. S6B). Moreover, silencing of KRAS abrogated coculture-induced EphA2 expression levels in HKH-2 cells, indicating that RAS activation is required for the EphA2 increases following coculture in KRASWT colorectal cancer cells (Fig. 4D). Clinically, a significant positive association between TGFα mRNA and EphA2 mRNA levels was observed in 4 independent colorectal cancer online datasets, further supporting the
hypothesis that stromal-derived growth factors can regulate EphA2 expression levels in an in vivo setting (Supplementary Table S5).

Discussion

While there have been major developments in the treatment of metastatic colorectal cancer over the last two decades (e.g., the introduction of the VEGF- and EGFR-targeted agents bevacizumab, aflibercept, ramucirumab, cetuximab, and panitumumab), translating these advances to stage II/III disease has shown no benefit in large phase III adjuvant clinical trials (1, 2). An improved understanding of the molecular mechanisms driving colorectal cancer progression and recurrence will potentially lead to the identification of novel diagnostics and treatment strategies for stage II/III colorectal cancer. In this study, we provide evidence that EphA2 is an important mediator of colorectal cancer cell migration/invasion and could be a promising novel target for colorectal cancer, in particular for stage II/III colorectal cancer with high EphA2 expression levels.

Table 2. EphA2 is an independent prognostic biomarker. A, univariate and multivariate analyses in stage II/III surgery-only colorectal cancer cohort (Singapore dataset) using the Cox Proportional Hazards Ratio method. B, final multivariate model (Cox proportional hazards regression) of surgery-only patient group in Singapore dataset. C, multivariate model (Cox proportional hazards regression) of GSE17536 patient group (stage II/III).

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| Factor                        | Multivariate (final) | C.
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In vivo, vivo

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in a decreased cancer stem cell population and tumorigenicity. EphA2 or EFNA1-FC (a soluble EFNA1 dimer fused to Fc) resulted respectively (10, 30). In addition, RNAi-mediated depletion of EphA2 and stem cell markers ALDH and DDEA-1a or CD44, and glioblastoma have shown a positive correlation between EphA2 and stem cell–like properties, and both markers have been associated with increased colorectal cancer tumorigenicity and metastasis (40, 41). Our study showed that high EphA2 expression significantly correlated with high expression levels of CD44 and Lgr5 in colorectal cancer tissues. Furthermore, shRNA-mediated knockdown of EphA2 suppressed the CD44-high stem-like colorectal cancer cell population, indicating that EphA2 may be a promising target to prevent colorectal cancer recurrence and metastasis.

In order to model colorectal tumor cell invasion/metastasis, our group has previously generated invasive (KRASMT/KRASWT/+chr3) colorectal cancer daughter cells which displayed an epithelial–mesenchymal transition–like phenotype, high levels of CD44, and increased colony-forming ability (17). We now show that EphA2 is highly expressed in these invasive daughter cell lines and that si/shRNA-mediated knockdown of EphA2 potently inhibited migration and invasion of parental and invasive colorectal cancer cells. In agreement with previous studies, we found that EphA2 was rapidly internalized and downregulated in a proteasome–dependent manner in response to EFNA1–ligand stimulation and this resulted in potent inhibition of migration in a panel of colorectal cancer cells (42). These data would indicate that EphA2 is an important regulator of migration and invasion in colorectal cancer.

A key finding of our study is that high EphA2 expression was significantly associated with poorer OS in stage II/III colorectal cancer. Importantly, further univariate and multivariate analyses revealed that the stage II/III surgery–only colorectal cancer patient cohort with high EphA2 expression levels has a shorter 5-year OS compared with stage II/III surgery–only colorectal cancer patients low EphA2 expression. The results herein obtained from both publicly available datasets and our colorectal cancer TMA are the first to show that EphA2 may be a biomarker of poor prognosis in stage II/III colorectal cancer. A previous RNA sequencing study of 675 human cancer cells has suggested that EphA2 is strongly correlated with the expression of other oncogenes, such as c-MET and EGFR (43). It is therefore plausible that analysis of c-MET and/or EGFR together with EphA2 can further enhance the prognostic power of EphA2 in stage II/III colorectal cancer.

Given its potential role in colorectal cancer progression and metastasis, we further investigated how EphA2 expression levels are regulated in colorectal cancer. In contrast with previous studies, our data did not show a role for p53 or the Src family kinases in regulating EphA2 expression (44, 45). Using a systems biology approach, we previously identified EphA2 as a potential KRAS target in KRASMT colorectal cancer cells (15). In agreement with previous studies in breast cancer and melanoma, our examination of a large panel of colorectal cancer cells revealed high EphA2 expression in cells harboring an activating mutation in KRAS or BRAF compared with KRAS/BRAFWT cells (28, 46).

Inhibition of the MAPK pathway resulted in significant increased EFNA1 mRNA expression while reducing EphA2 mRNA and protein levels. These data and our results using RNAi-mediated knockdown of EFNA1 or recombinant EFNA1 would indicate that expression of EFNA1 contributes, at least in part, to EphA2 levels in colorectal cancer, consistent with previous findings in breast cancer (28).

Activated Ras-GTP can exert its function through multiple downstream effectors, such as the Raf kinases, the p110 catalytic subunits of class I PI3Ks, PI3M, PLCe, and the RalGDS–Ral effector pathway (47). This is the first study showing that KRAS also regulates mRNA and protein levels of EphA2 through the RalGDS–Ral pathway. The Ras-like small GTPases RaLa and RaLb interact with effectors such as Sec5, Filamin, RALBP1, and ZONAB and have been shown to regulate membrane trafficking, cell adhesion, and transcription. A number of studies have shown that RaLa and RaLb are important drivers of survival, proliferation, and metastasis in solid tumors, including colorectal cancer (48). Using RNAi against RaLa and RaLb, we found that RaLa but not RaLb regulated EphA2 expression levels and migration in KRASMT colorectal cancer cells. These results would indicate that anti-RaL-selective therapies (49) may provide an effective therapeutic approach for KRASMT colorectal cancer with high expression levels of EphA2.

In contrast with the in vitro data, no correlation between the KRAS mutational status and EphA2 levels was found in our clinical samples. However, our data would suggest that microenvironment–derived ligands can regulate EphA2 levels in KRASWT tumors, which could explain why EphA2 levels were not significantly higher in the KRASMT colorectal cancer samples.

In conclusion, using preclinical colorectal cancer models and patient tissue samples, we have identified EphA2 as a key regulator of colorectal cancer cell migration and invasion. Moreover, EphA2 is highly expressed in colorectal cancer, associated with the colorectal cancer stem cell markers CD44 and Lgr5 and is a poor prognostic biomarker in colorectal cancer. A number of recent studies have identified the stem-like subtype as a dominant molecular subgroup with poor outcome in colorectal cancer (50). Our data would indicate that EphA2-targeted approaches may represent a promising treatment strategy for this stem-like subgroup with high EphA2 levels, in particular in the adjuvant disease setting where anti-EGFR agents have failed. A variety of therapeutic strategies have been developed to target EphA2, including activating monoclonal antibodies, ephrin ligands, and selective kinase inhibitors (3). Finally, our data provide support for the further investigation of EphA2 as a novel biomarker in early-stage colorectal cancer.

Disclosure of Potential Conflicts of Interest
R.H. Wilson is a consultant/advisory board member for Merck Serono and Sanofi. P.G. Johnston holds ownership interest (including patents) in CV6 Therapeutics and is a consultant/advisory board member for Chugai Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.D. Dunne, S. Dasgupta, J.K. Blayney, T. Sasazuki, S. Shirasawa, K. Arthur, P.G. Johnston, S. Van Schaeybroeck

Study supervision: P.D. Dunne, R.H. Wilson, P.G. Johnston, S. Van Schaeybroeck

Other (pathologic scoring on biopsies with IHC staining): T. Wang

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