ORIGINAl ARTICLE

Integrated genomic analysis of colorectal cancer progression reveals activation of EGFR through demethylation of the EREG promoter

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Key molecular drivers that underlie transformation of colonic epithelium into colorectal adenocarcinoma (CRC) are well described. However, the mechanisms through which clinically targeted pathways are activated during CRC progression have yet to be elucidated. Here, we used an integrative genomics approach to examine CRC progression. We used laser capture microdissection to isolate colonic crypt cells, differentiated surface epithelium, adenomas, carcinomas and metastases, and used gene expression profiling to identify pathways that were differentially expressed between the different cell types. We identified a number of potentially important transcriptional changes in developmental and oncogenic pathways, and noted a marked upregulation of EREG in primary and metastatic cancer cells. We confirmed this pattern of gene expression by in situ hybridization and observed staining consistent with autocrine expression in the tumor cells. Upregulation of EREG during the adenoma–carcinoma transition was associated with demethylation of two key sites within its promoter, and this was accompanied by an increase in the levels of epidermal growth factor receptor (EGFR) phosphorylation, as assessed by reverse-phase protein analysis. In CRC cell lines, we demonstrated that EREG demethylation led to its transcriptional upregulation, higher levels of EGFR phosphorylation, and sensitization to EGFR inhibitors. Low levels of EREG methylation in patients who received cetuximab as part of a phase II study were associated with high expression of the ligand and a favorable response to therapy. Conversely, high levels of promoter methylation and low levels of EREG expression were observed in tumors that progressed after treatment. We also noted an inverse correlation between EREG methylation and expression levels in several other cancers, including those of the head and neck, lung and bladder. Therefore, we propose that upregulation of EREG expression through promoter demethylation might be an important means of activating the EGFR pathway during the genesis of CRC and potentially other cancers.

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INTRODUCTION

The development of colorectal cancer (CRC) is known to proceed through the acquisition of genetic alterations during disease progression.1 In colonic adenomas, there is disruption of the function of tumor suppressor gene, APC, as well as activating mutations in oncogenes, such as KRAS. Later events during the transition to carcinoma include loss of tumor suppressor gene, TP53.1 As significant progress has been made towards understanding the biology of normal colonic epithelium,2,3 it has become evident that many of the pathways regulating normal colonic surface and crypt homeostasis are also involved in oncogenic transformation, including the WNT, NOTCH, TGF-β (transforming growth factor-β), MAPK (mitogen-activated protein kinase) and PI3K/AKT (phosphatidylinositol 3-kinase/AKT) pathways.3-10 Genomic aberrations, including mutations, microsatellite instability (MSI) and chromosomal instability (CIN) are known drivers of colon epithelial transformation, and DNA methylation also contributes to disease development.2,4,11-14 This has led to two molecularly defined subsets of CRC being described in recent years. One includes the CpG island methylator phenotype (CIMP) subtype that often exhibits microsatellite instability (MSI), a high frequency of BRAF mutation and represents ~15% of CRC.14 The other subset is defined by CIN/TP53 that frequently carries KRAS mutations and accounts for ~85% of CRCs.14

While CIMP and CIN/TP53 subtypes encompass molecular events of significance in CRC, activation of receptor tyrosine kinase signaling has also been shown to have an important role in driving colon carcinogenesis and associated angiogenesis.5,14 Indeed, the two classes of clinically approved therapies in CRC are antagonists of the vascular endothelial growth factor/receptor-2 (VEGF/VEGFR2) and epidermal growth factor receptor (EGFR) receptor tyrosine kinase signaling pathways, both of which are typically used in combination with fluorouracil-containing chemotherapy.16-18 Patients with RAS/RAF mutant tumors do not usually respond well to EGFR-targeted therapies but do experience clinical benefit when treated with antiangiogenic drugs, such as...
as avastin.\textsuperscript{19–22} Conversely, patients with RAS/RAF wild-type tumors have been shown to respond favorably to EGFR antagonistic antibodies, such as cetuximab.\textsuperscript{19–21,23} Retrospective analyses have also suggested that patients with RAS wild-type tumors that express high level of the EGFR ligands, EREG and AREG, might benefit from cetuximab treatment.\textsuperscript{11,24,25} However, the timing and mechanism through which the EGFR pathway is activated during CRC progression have yet to be revealed.

In this study, we examined CRC progression using an integrative genomic approach. We observed broad transcriptional differences between laser capture-microdissected (LCM) normal colonic surface epithelium, crypt cells, adenomas and CRCs in pathways known to be involved in cell proliferation, differentiation and transformation. Here, we focused on the clinically relevant EGFR pathway because of the marked upregulation of the gene encoding for the EGFR ligand, EREG, that we observed at the adenoma–carcinoma transition. Mechanistically, we found EREG-mediated activation of the EGFR pathway in CRC to be associated with demethylation of its promoter. We demonstrated in CRC cell lines that global demethylation released epigenetic inhibition of EREG and led to higher levels of EGFR phosphorylation, as well as increased sensitization to EGFR inhibitors. In patients who received cetuximab as part of a phase II trial, we observed low levels of EREG methylation and high level of ligand expression in tumors that exhibited the best responses. Finally, we detected an inverse correlation between EREG methylation and expression levels in different tumor types, suggesting that epigenetic regulation of EREG expression might be a common mechanism for EGFR pathway activation in several types of malignancies.

**RESULTS**

An integrative molecular view of colorectal cancer progression

To gain a molecular understanding of normal colonic epithelial biology and CRC progression, we used an integrative genomics approach. First, we used LCM to isolate cells from normal colonic crypts ($n=7$), normal colonic surface epithelium ($n=6$), colonic adenomas ($n=17$), primary colorectal carcinomas ($n=17$) and CRC distant metastases ($n=11$) (Figure 1a and Supplementary Table S1). To obtain a broad view of the molecular patterns from normal and pathophysiological cell types, we performed gene expression profiling analysis. Because of the limited amount of material available from these samples, we performed targeted next-generation sequencing, genome-wide methylation and reverse-phase protein array (RPPA) analyses on a series of 48 macrodissected frozen samples of normal colonic epithelium ($n=14$), adenomas ($n=12$), primary colorectal

![Figure 1. Integrative analysis of colorectal cancer progression. (a) Summary of sample characteristics and assays run on each sample. (b) Hierarchical clustering of the gene expression scores for the top 500 most variable genes across all samples (after adjustment for one surrogate variable). Below heatmap: Examples of LCM cells from normal surface epithelium, colon crypts, adenomas, carcinomas and metastases. (c) Selected differentially expressed signaling pathways between normal surface and normal crypt, and/or between adenoma and carcinoma, and differentially expressed genes annotated as ERBB signaling components by Ingenuity analysis ($P<0.05$, log 2 fold change >0.5). Predicted direction of pathway activation based on Ingenuity analysis is reflected by a +/− sign on the respective bars. (d) Bar plots representing expression levels of ERBB family ligands and receptors in adenomas, carcinomas and metastases. Two-sided $P$-values were derived using an unpaired $t$-test. \textit{****} $P<0.00005$.](Image)
carcinomas (n = 13) and CRC metastases (n = 9) (Figure 1a and Supplementary Table S2). To establish a transcriptional view of normal and malignant colonic development, we used Affymetrix microarrays and profiled LCM tissue samples. Unsupervised hierarchical clustering based on the expression of 500 genes that exhibited the most variation in expression across all samples revealed a number of notable features. Overall, samples segregated into two major groupings: (1) carcinomas and metastases interspersed with one another, and (2) normal crypts cells, surface epithelial cells and adenomas (Figure 1b). Although crypt and surface epithelial cells cosegregated, they showed some of the most striking differences in gene expression, as clearly demonstrated by principal component analysis (Figure 1b and Supplementary Figure S1), reflecting undifferentiated cells within the crypt environment and differentiated surface epithelial. That carcinomas and metastases showed a lack of significant differences in gene expression (as judged by their cosegregation) (Figure 1b) is consistent with the concept that the time from carcinoma and metastasis is significantly shorter (< 2 years) than the time interval from adenoma to carcinoma (~17 years), and is suggestive of few additional alterations occurring during metastatic seeding and growth.

To understand the key molecular features underlying the differences in cell types, we used the 1416 differentially expressed genes between normal surface epithelium and crypt cells, and 643 differentially expressed genes between adenomas and carcinomas at a false discovery rate of < 5% and absolute log 2 fold change > 0.58 (Supplementary Table S3), and queried them in the canonical pathway annotation in Ingenuity analysis. Several pathways known to be involved in colonic cell differentiation were differentially expressed between surface epithelium and the crypt compartments, including the WNT/β-catenin, TGF-β and G1/S cell cycle checkpoint, as well as the mTOR, TP53 and DNA methylation and double-stranded break repair pathways (Figure 1c and Supplementary Table S4). At the adenoma–carcinoma transition, genes representative of the G2/M checkpoint and PI3K/AKT pathways were more prominently differentially expressed (Figure 1c). Interestingly, genes belonging to the ERBB pathway exhibited a mixed pattern of expression that indicated preferential activation of the ERBB2 pathway in surface epithelium compared with crypt cells, and a similar degree of neuregulin pathway activation in surface/crypt and adenomas/carcinomas (Figure 1c), suggesting that ERBB pathway may have a role in both normal colon epithelial cell differentiation and malignant transformation.

We used a custom next-generation sequencing cancer panel to assess the mutational status of CRC-relevant genes in our progression sample set, and detected mutations in APC, KRAS, PIK3CA, PTEN, SMAD4 and TP53 (Supplementary Figure S2). The temporal occurrence of mutations was consistent with the reported timing of these genetic alterations during CRC progression. For example, we noted the presence of APC and KRAS mutations in adenomas, whereas TP53 mutations were detected in carcinomas (Supplementary Figure S2). Thus, our targeted next-generation sequencing data recapitulates the presence and timing of previously described mutations, and suggests that our cohort is suitable for discovery of molecular alteration associated with the genesis of CRC.

As depicted in Figure 1c, we identified multiple pathways that showed differential expression between the different tissue types; however, given the therapeutic importance of EGFR in CRC, we examined the expression of members of this pathway in our LCM tissues. Several genes encoding EGFR pathway components were differentially represented between surface epithelium and crypt cells, including the ligands AREG and TGF-α, the ERBB3 receptor, as well as downstream components such as SHC, NCK1, NCK2, c-Fos and c-Jun (Figure 1c). Of these genes, only AREG and NCK1 exhibited differential expression between adenoma and carcinomas, and AREG was upregulated specifically in carcinomas (Figure 1c). A closer examination of the expression levels of ERBB family ligands and receptors in adenomas compared with carcinomas and metastases showed no significant increase in any of the four receptors and most of the ligands, and a marginal, but not significant, increase in the levels of TGF-α in adenomas compared with carcinomas and metastases (Figure 1d). On the other hand, AREG and EREG were expressed at significantly higher levels in carcinomas versus adenomas (Figures 1c and d and Supplementary Table S3). Interestingly, EREG was upregulated in carcinomas compared with adenomas but was not differentially expressed between colonic surface epithelium and crypt cells (Figures 1c and d and Supplementary Table S3), pointing to the possibility that AREG and EREG might be having different roles in normal colonic epithelial cell differentiation and in CRC development.

EREG transcript is detected at low levels in non-malignant cells and its levels are markedly increased in carcinomas. Because we specifically isolated adenomatous and tumor cells by LCM, we hypothesized that expression of EGFR ligands likely occurred in a cell-autonomous manner. To test this hypothesis, we performed in situ hybridization (ISH) analysis using a custom assay on tissues from an independent-sample set (Supplementary Table S5). EREG transcript number and localization were quantified in matched normal, adenoma and carcinoma tissues from the same patients (see Materials and methods). A representative image showing EREG transcript signals in a case that included normal colonic epithelium, adenoma and carcinoma histologies in the same tissue section is shown in Figure 2a. We found that normal colonic epithelial cells and adenomas showed low levels of EREG (Figure 2a). In contrast, a neighboring carcinoma exhibited significantly higher levels of EREG specifically in tumor cells (Figure 2a). Stromal cells showed minimal EREG ISH signal, irrespective of whether they were adjacent to normal epithelium, adenomas or carcinomas (Figures 2a and b). In the remaining samples, we observed that the EREG signal was low to absent in most normal colonic epithelia, stromal cells and adenomas, but was markedly higher in a majority of carcinomas (Figure 2b).

We also examined the levels and localization of the AREG transcript in the same cohort using a custom ISH assay. We found that AREG signals were significantly higher in carcinomas compared with adenomas (Supplementary Figure S3). However, unlike in the case of EREG, where expression was high in tumor cells and low to absent in adenomas and normal colonic epithelium, a wide range of AREG ISH signal was detected in normal colonic epithelia and in adenomas that was, in some cases, comparable to the levels typically observed in carcinomas (Supplementary Figure S3). AREG signal was not detected in stromal cells (Supplementary Figure S3). The observation that EREG was markedly upregulated and expressed specifically in tumor cells is consistent with the EGFR ligand as a potential driver of CRC development, and supports an autocrine model of pathway activation that has been proposed in head and neck and lung cancers.

EREG promoter demethylation and transcriptional upregulation are associated with increased EGFR phosphorylation during the adenoma–carcinoma transition

Epigenetic regulation of gene expression has been shown to have an important role in CRC development. To gain a broad view of genes that might be epigenetically regulated during CRC development, we examined the promoter methylation status within the promoter region 2 kb downstream to 0.5 kb upstream of transcriptional start sites in macrodissected tissues. In total,
gene expression and promoter methylation data was available for 11,813 genes in 34 samples. We calculated Spearman’s rank correlation between gene expression and methylation data across matched adenomas, carcinomas and metastases to identify genes that might be regulated by promoter methylation (Supplementary Table S6). Interestingly, the MAPK pathway activation suppressor, dual specificity phosphatase 4, was the top-scoring gene, exhibiting the highest negative correlation between promoter methylation and gene expression (Figure 3a). Other genes that exhibited the strongest inverse correlations between their levels of methylation and expression included Disabled-2, which is often silenced in gastrointestinal tumors, S100 calcium binding protein A4, which has been shown to interact with and degrade TP5336 (Figure 3a). We found EREG to be one of the top-ranked genes that exhibited a large negative correlation coefficient (Figure 3a and Supplementary Table S6, 28 out of 11,813 genes), suggesting that the upregulation of EREG expression during CRC progression might be mediated by promoter demethylation.

To determine the sites responsible for epigenetic regulation of EREG expression in CRC, we examined the methylation status for all eight available probes within the promoter region. Among these two probes, cg11646192 and cg19308222, spaced 224bp apart, showed a strong inverse correlation between EREG expression and promoter methylation, and were highly correlated with each other (Figures 3b and c). The six remaining probes were consistently either hyper- or hypomethylated in all samples irrespective of the tissue type and levels of EREG expression (Figure 3b). We examined the methylation signals of the cg11646192 and cg19308222 probes in our CRC progression samples and found low levels of methylation in normal colon surface epithelium and crypt tissue, a high degree of methylation in adenomas and demethylation in primary tumors and metastases (Supplementary Figure S4). These data suggest that increased EREG expression during CRC development might be due to promoter demethylation at these two positions. Although the expression of both EREG and AREG was higher in carcinomas and metastases compared with that in adenomas, the methylation status of two AREG methylation probes did not show a significant correlation with its expression levels (Supplementary Figure S5).

We next asked if decreased methylation and increased EREG expression had an impact on the phosphorylation status of two key sites on the EGFR receptor (EGFR-Y1068 and -Y1173). These two sites have been shown to be phosphorylated and activated by EGFR ligands.37,39 Using RRPA, we observed significantly higher levels of EGFR phosphorylation in carcinomas and metastases compared with adenomas at both sites (Figure 3d), consistent with EGFR signaling being activated at the adenoma–cancer transition and maintained in distant CRC metastases. Moreover, a significant inverse correlation between EREG promoter methylation and EGFR phosphorylation was also observed at both sites in adenomas, carcinomas and metastases (Figure 3e). Taken together, our data suggest that activation of EGFR signaling is highly correlated with demethylation of specific sites within the EREG promoter and concomitant upregulation of the ligand during CRC progression.

Validation of the inverse correlation between EREG methylation and expression during CRC development

To validate the relationship between EREG demethylation and expression during CRC progression, we used an independent set of samples from 16 patients with matched adenomas and carcinomas (Supplementary Table S5). We measured the percentage of EREG promoter methylation using a custom methylation-specific PCR assay,38 and assessed EREG expression levels using a commercial TaqMan Real-Time PCR assay (see Materials and methods). Consistent with our observations in the discovery cohort, EREG methylation was higher in adenomas compared with matching carcinomas, with concomitantly higher ligand expression levels in a subset of carcinomas compared with that in matched adenomas (Figure 4a). Notably, EREG expression was highest in carcinomas with the lowest levels of promoter methylation (Figure 4a). In aggregate, we detected significantly higher levels of EREG promoter methylation in adenomas compared with carcinomas (Figure 4b), and significantly higher transcript levels in carcinomas versus adenomas (Figure 4c). These results independently confirm that a decreased state of EREG methylation is often associated with increased levels of ligand expression during the adenoma–cancer transition.

Given that CIMP defines a distinct molecular CRC subtype that is known to be associated with response to 5-fluorouracil-based therapies,44,45,46 we next examined the relationship between CIMP status and EREG methylation in CRC tissues from the Cancer
significantly higher in CIMP+ compared with CIMP−. **P<0.005, ***P<0.0005 and NS, not significant.

Genome Atlas Network (TCGA) collection. Because CIMP status was not available for all samples and given the high degree of concordance between CIMP status and MLH1 promoter methylation that has been described previously,14 we used MLH1 promoter methylation as a surrogate for CIMP status (see Materials and methods). As expected, EREG methylation levels were significantly higher in CIMP+ compared with CIMP− samples (Supplementary Figure S6a). Consistently, EREG expression levels were significantly lower in CIMP+ compared with CIMP− samples (Supplementary Figure S6b). A similar relationship between CIMP status and AREG expression was also observed (Supplementary Figures S6c and d). Interestingly, ~25% of CIMP− samples exhibited high levels of methylation and low levels of EREG expression (Supplementary Figures S6a and b), suggesting that epigenetic regulation of EREG can occur in CIMP− samples and that epigenetic control of EREG cannot be explained solely by CIMP.

Our validation studies in an independent set of matched adenomas and carcinomas and from TCGA tissues are consistent with our finding that EREG expression can be epigenetically regulated during CRC progression, and suggest that this phenomenon can occur in both CIMP+ and CIMP− disease.

Demethylation and transcriptional upregulation of EREG leads to EGFR activation and sensitizes CRC cell lines to EGFR inhibitors. To begin to assess the role of methylation in regulating EREG expression and EGFR signaling, we next examined the expression and methylation status of EREG in a panel of CRC cell lines. Consistent with what we observed in the discovery and independent validation tissues sets, we noted an inverse correlation between EREG expression and methylation cross 40 CRC cell lines (Supplementary Figure S7a and Supplementary Table S7). From these 40 cell lines, we selected a representative panel of 10 CRC cell lines that exhibited low, intermediate and high basal levels of EREG expression and, accordingly, high, intermediate and low basal levels of EREG methylation (Figure 5a). We next assessed the basal EGFR phosphorylation levels in these 10 cell lines at the EGFR-Y1068 and -Y1173 sites that are known to be indicative of receptor activation.37–39 We found cell lines with
high basal levels of EREG methylation and low basal levels of expression (Colo302DM, Colo741 and RKO) to have barely detectable levels of EGFR phosphorylation at both sites (Figure 5c). In contrast, EREG phosphorylation was readily detectable in cell lines with intermediate levels of EREG expression and methylation (C28Be1, CL11 and HCA7) and in cell lines with low basal levels of EREG methylation and intermediate/high EREG expression levels (HCT15, SW48, HCT116 and in DLD-1) (Figures 5a–c). These findings support a strong association between basal EREG methylation and expression status and EGFR phosphorylation levels in CRC cell lines.

To begin assessing the functional consequences of EREG methylation on EGFR phosphorylation in CRC cell lines, we treated the 10 representative CRC cell lines with the DNA demethylating agent, 5-aza-dC. Following treatment, EREG methylation was significantly reduced in cells with high basal levels (Colo320DM, Colo741, RKO) and with intermediate basal levels (C28Be1, CL11 and HCA7) of EREG methylation (Figure 5a). A significant increase in EREG expression levels was observed in all cell lines with the exception of HCA7, which exhibited a marginal increase of EREG expression (Figure 5b). In contrast, no induction of EREG expression was observed in cells with high basal levels of EREG expression (HCT15, SW48, HCT116 and DLD-1) (Figure 5b). After 5-aza-dC treatment, we also observed an increase in the levels of EGFR phosphorylation at both Y1068 and Y1173 sites in Colo741, RKO, C28Be1 and CL11 cells (Figure 5c). No increase in EGFR tyrosine phosphorylation was observed in HCT15, SW48, HCT116 and DLD-1 cells (Figure 5c). These results suggest that DNA methylation most likely has an important role in regulating EREG expression and EGFR phosphorylation in CRC cell lines.

We next sought to determine whether the induction of EREG expression after 5-aza-dC treatment and the resulting increase in EGFR phosphorylation would enhance the sensitivity of CRC cell lines to the EGFR inhibitors, gefitinib and erlotinib. Unfortunately, very few CRC cell lines have been reported to be responsive to EGFR antagonists in vitro. Among the four cell lines with high levels of endogenous EREG expression and EGFR phosphorylation, we chose SW48 as a control because it was the only sensitive line to treatment with EGFR inhibitors (Figure 5d and Supplementary Figure S7b). We also selected cell line CL11 because it exhibited phospho-EGFR induction after 5-aza-dC treatment, which was comparable to the levels observed in SW48 cells. We pretreated SW48 and CL11 cells with 5-aza-dC for 2 days and then added 0.11, 0.33 or 1 μM of EGFR inhibitor alone or in combination with 5-aza-dC. Interestingly, a significant decrease of cell viability was observed in CL11 cells, which were treated with the combination of gefitinib and 5-aza-dC compared with those treated with gefitinib alone (Figure 5d). In contrast, no decrease in cell viability was observed in SW48 cells (Figure 5d). Similar results were observed in response to treatment with erlotinib (Supplementary Figure S7b). These data suggest that induction of EREG expression and EGFR phosphorylation after treatment of CL11 cells with 5-aza-dC sensitizes these cells to the EGFR inhibitors, gefitinib and erlotinib.

EREG methylation in CRC is associated with lack of clinical response to cetuximab treatment and may regulate EREG expression in several other types of cancers

EREG expression has been reported to predict clinical benefit in response to anti-EGFR treatment. As we demonstrated that upregulation of EREG expression during CRC progression was associated with demethylation of its promoter in both our discovery and independent validation sets, we hypothesized that low methylation levels of EREG in tumors should predict favorable clinical response to cetuximab. To test this hypothesis, we analyzed primary tumor tissues from CRC patients with KRAS wild-type tumors who received cetuximab+FOLFOX in the second-line metastatic setting as part of the MEHD7945A phase II clinical trial. We evaluated the methylation and expression levels of EREG.

**Supplementary Information**

Figure 4. EREG expression and methylation in an independent patient set with matched adenoma and carcinoma tissues. (a) Percentage of EREG methylation using a custom assay, and relative EREG expression using a commercial Taqman assay in a series of matched adenomas and carcinomas. (b) Significantly higher levels of EREG methylation were observed in adenomas compared with carcinomas. (c) Significantly lower levels of EREG expression were detected in adenomas compared with carcinomas. Two-sided P-values were derived using a paired t-test, n = 16. ***P < 0.001 and ****P < 0.0001.

Figure S7. Cytotoxicity assays. (a) Treatment with 5-aza-dC sensitizes these cell lines to the EGFR inhibitors, gefitinib and erlotinib. (b) Inhibition of cell viability in CRC cell lines in response to treatment with the EGFR inhibitors, gefitinib and erlotinib. (c) Inhibition of cell viability in CRC cell lines in response to treatment with the EGFR inhibitors, gefitinib and erlotinib.
in tissues from 33 patients (Supplementary Table S8). Waterfall plots of best % helical computed tomography (CT) responses compared with baseline tumor measurements showed that six of seven (86%) of the patients who exhibited ≥50% tumor size reduction had low levels of EREG promoter methylation (Figure 6a). Conversely, five of six (83%) of the patients whose tumors grew after treatment exhibited high levels of methylation (Figure 6a). Consistent with our earlier observations from the discovery and validation cohorts, EREG expression in samples from the phase II trial was inversely correlated with levels of promoter methylation (Figure 6b). Importantly, low levels of EREG promoter methylation were significantly correlated with tumor size reductions (Figure 6c). In line with these findings, increased EREG expression positively correlated with cetuximab activity (Figure 6c). Although AREG and EREG expression were significantly correlated in samples from the phase II study (Supplementary Figure S8a), AREG levels were not significantly associated with tumor size reduction following cetuximab+FOLFIRI treatment (Supplementary Figure S8b).

Increased levels of EREG have been reported in other cancer types, however, the mechanism through which the ligand is regulated in these indications is largely unknown. To investigate whether methylation might be a mechanism for EREG regulation in cancers beyond CRC, we examined the relationship between EREG expression and methylation in samples from the TCGA data set.43 We observed an inverse correlation between EREG expression and methylation in various cancer types, and, as expected, this was most notable in rectal and colon cancer (Figure 6d). Interestingly, head and neck cancers, lung adenocarcinomas, acute myeloid leukemias and squamous cell lung cancers all exhibited inverse correlations, indicating that EREG methylation might be regulating gene expression in these cancer types. Notably, these are indications in which anti-EGFR therapies have demonstrated antitumor activity.40,45,47–49 In contrast, we observed a weaker inverse correlation between AREG methylation and expression across the TCGA indications (Supplementary Figure S9). Our data from the analysis of MEHD7945A samples and TCGA data sets suggest that EREG demethylation might be a mechanism for activating the EGFR pathway in CRC and, potentially, other types of cancers.

**DISCUSSION**

Our integrative molecular characterization of normal colonic surface epithelium, crypt cells, adenomas, primary carcinomas and distant metastases detected known and identified novel genetic drivers of CRC. Using targeted next-generation sequencing, we confirmed the presence of mutations in APC and KRAS genes as occurring early during the normal–adenoma transition, and TP53 mutations as occurring late in CRC development.1,26 On the transcriptional level, use of LCM enabled us to show that normal colonic surface epithelium and crypt cells are transcriptionally distinct from carcinomas and metastases, exhibiting wide-ranging
Figure 6. EREG methylation in CRC is associated with lack of response to cetuximab treatment and might be a mechanism for regulating EREG expression in several other cancer types. (a) Waterfall plot of % best CT response and associated EREG methylation and expression in samples from the cetuximab arm of an MEHD7945A+FOLFIRI phase II clinical trial. A median cutoff was used to designate samples as having high vs low % methylation or expression. (b) EREG methylation is inversely correlated with its expression in samples from the phase II clinical trial; n = 33 samples. Pearson’s R = −0.79. (c) Association between EREG methylation and expression with % best CT response in the samples from the phase II clinical trial; n = 33 samples. Pearson’s R = 0.51 and −0.43, respectively. (d) Correlation between EREG promoter methylation and expression across all TCGA indications with detectable EREG. AML, acute myeloid leukemia; BLCA, bladder urothelial carcinoma; BRCA, breast-cancer; BRIN, brain neoplasms; ESCA, esophageal cancer; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KIR1, kidney renal clear cell carcinoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MB, central nervous system meningeal neoplasms; MIBC, bladder urothelial carcinoma, muscle invasive; Mixed, other tissue types; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrioid carcinoma.

Differences from them in the expression of genes belonging to developmental pathways including G1/S cell cycle checkpoint, TP53, WNT, TGF-β and mTOR. Hierarchical clustering indicated that gene expression in the normal crypt cells is more akin to that of adenomas than surface colonic epithelium, supporting the notion that adenomas and the normal crypt compartment might share a common origin, with clonal expansion of adenomas possibly resulting from mutations in stem cells within the crypts. We found that carcinomas and metastases were transcriptionally similar, consistent with previous findings that molecular determinants of metastasis are already present in primary carcinomas.26

Despite exhibiting many transcriptional differences, some pathways were consistently differentially expressed between both surface and crypt epithelium and also between adenomas and carcinomas, including EGFR-related neuregulin, and the ERK/MAPK pathways. Interestingly, a closer examination of EGFR pathway components revealed that EREG and AREG had similar expression profiles in CRC but different patterns of expression in normal colonic epithelium. Although TGF-α was upregulated during the adenoma–carcinoma transition, this change was not statistically significant. There are several reports that point to potentially distinct roles for TGF-α, AREG and EREG in ERBB pathway signaling in cancer.26,50–52 In our data set, upregulation of EREG was observed in carcinomas compared with adenomas but not in surface epithelium compared with crypt epithelium, whereas AREG was consistently upregulated in both normal surface epithelium and in carcinomas. In line with our molecular data, examination of the spatial distribution of EREG by ISH showed minimal staining in normal surface and crypt epithelium, adenomas, and stromal cells, but strikingly high signals in carcinomas and metastases. Thus, the production of EREG by CRC cells appears to occur in an autocrine manner. Although AREG expression levels were significantly higher in tumors compared with other tissue types, AREG staining patterns were not tissue-specific, and the wide range in AREG signal intensity in normal colonic epithelium and adenomas was overlapping with that observed in carcinomas.

When we integrated genome-wide methylation and expression data to identify genes that might be epigenetically regulated, we found that EREG was one of the genes with the highest inverse correlation between methylation and expression levels. These findings were consistent with epigenetic silencing of EREG through methylation of the promoter in adenomas. EREG methylation was reversed during the transition to carcinoma, and this was associated with an upregulation of the ligand in cancers. In analyzing samples from our validation set of matched adenomas and carcinomas, we observed high levels of methylation and minimal expression of EREG in adenomas, whereas many matched carcinomas from the same patients exhibited low
Cancers.50,53 Colitis-associated neoplasms and potentially other types of tumors to exhibit high levels of EREG methylation and low levels of EGFR pathway activation. It is possible that epigenetic control of EREG might represent a targeted mechanism for EGFR pathway activation in CRC. In tissues, we observed a strong inverse correlation between EREG expression and phosphorylation of EGFR in adenomas. This result is consistent with previous studies that have demonstrated a relationship between EREG expression and EGFR pathway activation in CRC. In our study, we found a significant association between EREG expression and EGFR phosphorylation in adenomas, with higher levels of EREG expression associated with a concomitant increase in EGFR phosphorylation levels. This finding is consistent with previous studies that have shown a correlation between EREG expression and EGFR phosphorylation in CRC.

To begin to assess the clinical consequences of EREG methylation, we examined the relationship between EREG demethylation and expression levels in CRC. We found that tumors that exhibited the best CT responses after therapy were those with the lowest levels of EREG expression. Conversely, tumors that grew following cetuximab treatment exhibited high levels of EREG expression. These data provide clinical support for a mechanism of EGFR pathway activation during CRC development through EREG promoter demethylation. Studies have previously shown that a positive CIMP status can lead to a diminished response to 5-fluorouracil-containing therapies in metastatic CRC.51,52 Thus, it is possible that CIMP status did have an impact on response to treatment in our single arm cohort. However, we do not believe that this fully explains the association we observed between EREG methylation and clinical response to therapy. Further studies are needed to investigate the role of epigenetic regulation of EREG in CRC and its potential as a therapeutic target.
response in our phase II study, in part, because EREG methylation did not always coincide with CIMP+ status, as we observed high levels of EREG methylation (and low levels of expression) in a subset of CIMP− samples from the TCGA collection. The EGFR antagonist, cetuximab, likely also had a role in contributing to the CT responses in our cohort. It is noteworthy that some patients from our phase II cohort whose tumors had high levels of EREG methylation (some of which would be expected to be CIMP+) did exhibit clinically meaningful CT responses. This may suggest that several predictive factors are at play, including EREG, CIMP status and potentially others that we are not aware of at this point in time.

Our finding that EREG expression can be epigenetically regulated in a subset of CRC raised the possibility that this mechanism might also be relevant in other cancer indications. We investigated the relationship between EREG promoter methylation and expression in available TCGA data sets. We found an inverse correlation between EREG methylation and expression levels in several types of malignancies. Most of these cancers were ones in which EGFR-targeting therapies have demonstrated clinical activity, such as neoplasms of the head and neck, lung and bladder. Interestingly, not all of these cancer types exhibited this relationship between EREG methylation and expression, suggesting that EGFR pathway activation might proceed through other mechanisms in those tumor types. For example, increased methylation and inactivation of EREG is known to occur during the development of gastric cancer.

In this study, we provide data that support a model for EGFR pathway activation during the evolution of CRC that may be driven through demethylation and subsequent upregulation of EREG at the adenoma–carcinoma transition in a subset of tumors (Figure 7). Future studies will be required to elucidate this potential mechanism of EGFR pathway activity as a driver of tumor progression in CRC and other cancer types.

**MATERIALS AND METHODS**

**Patient and tissue samples**

The use of the discovery set of samples from the Department of Pathology at the University of Virginia was approved by their Institutional Review Board. Laser microdissection was performed using a Leica AS LMD system (Leica Microsystems Inc., Bannockburn, IL, USA). Clinical details of the discovery set of 58 laser-captured frozen tissue samples and 48 macropiedized frozen CRC samples can be found in Supplementary Tables S1 and S2, respectively. To our knowledge, samples were obtained before therapy; however, 2/17 carcinomas and 1/17 adenomas were of rectal origin and patients may have received neoadjuvant therapy before tissue resection. The validation set of 16 matched adenomas and carcinomas were obtained from the MT Group (Van Nuys, CA, USA) and had appropriate Institutional Review Board approval. Clinical characteristics of the validation set are shown in Supplementary Table S5. Information on samples from the cetuximab arm of the phase II MEHD7945 trial (NCT01652482) can be found in Supplementary Table S8. All tissues were subjected to review by a pathologist to confirm the diagnosis and to define each cell type-enriched areas for macrodissection. Total RNA was purified using High Pure FFPE (Formalin-Fixed, Paraffin-Embedded) Micro Kit (Roche Diagnostics, Indianapolis, IN, USA). Total DNA was prepared by QiAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) and DNeasy Blood and Tissue Kit (Qiagen), respectively.

**Next-generation sequencing**

Mutations were detected using a previously developed MMP-Seq targeted cancer panel. DNA sample quality was quantified as the number of functional copies using a TRAK2 qPCR ‘ruler assay’. Approximately 5000 functional copies of DNA from each sample were used as the input for target enrichment and library construction using Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA, USA), followed by deep sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). Sequence alignment, primary variant calling and filtering were performed as described previously.

**Gene expression analysis**

Analyses were performed using the R programming language (version 3.2). Gene expression profiles were collected on Affymetrix HG-U133A GeneChips (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s instructions. Gene expression data was deposited into the GEO database under the accession number GSE77953. Gene expression values were obtained by quantile normalization and calculation of the robust multichip average expression measure using the affy Bioconductor package (version 1.46.1). To account for potential confounding variables, for example, batch effects, surrogate variable analysis was performed with the sva Bioconductor package (version 3.14.0), which identified one variable that was included when fitting linear models (see below). For hierarchical clustering (Figure 1b), we fit the normalized gene expression values versus the surrogate variables and used the observed residuals to identify the top 500 genes with the largest interquartile range. Residual gene expression scores were transformed to z-scores and clustered based on a Pearson correlation as distance metric for Ward’s clustering method. Principal component analysis was performed using residual gene expression scores from all assayed probes and the first two components were plotted (Supplementary Figure S1, PC1 and PC2).

We identified genes displaying differential expression between successive stages of CRC progression by applying a moderated t-test using the limma Bioconductor package (version 3.24.12). To account for potential confounding variables, the surrogate variable identified above was included together with the CRC progression stage in the linear model. Successive stages were contrasted and genes with false discovery rate < 0.05 and a minimum absolute log 2 fold change of ≥ 0.58 (1.5-fold up- or downregulated) were considered significantly differentially expressed. Data were analyzed through the use of QIAGEN’s Ingenuity Pathway Analysis (QIAGEN, Redwood City, CA, USA; www.qiagen.com/ingenuity). Genes that were differentially expressed between normal colonic surface epithelium compared with crypt cells or between carcinomas compared with adenomas at a log 2 fold change of ≥ 0.58 were included in the pathway analysis (Supplementary Table S5). Pathway significance was measured as −log (P-value) and ranged from 0 to 38.5, and a list of pathways and associated significance is provided in Supplementary Table S4.

**Taqman real-time PCR assay**

The high-capacity cDNA Reverse Transcription Kit (cat. no. 4368814; Applied Biosystems, South San Francisco, CA, USA) was used to prepare cDNA from 200ng of total RNA. Relative cDNA quantification for EREG expression using GAPDH as an internal reference gene was carried out using Applied Biosystems Viia7 Real-Time PCR System (Thermo Fisher Scientific, South San Francisco, CA, USA) following the standard protocol of the manufacturer. The primers and probe sets for each gene are from Life Technologies (South San Francisco, CA, USA) and associated significance is provided in Supplementary Table S4.

**Illumina inфинium analysis**

Genome-wide DNA methylation analysis was performed on DNAs from 48 fresh-frozen samples. Microarray data were collected at Expression Analysis Inc. (Durham, NC, USA) using the IlluminaHumanMethylation450 Beadchip (Illumina) and preprocessed using the Bioconductor methylumi software package (version 2.14.0; PMID 18467348) as described previously.

Genome-wide methylation data were deposited into the GEO database under the accession number GSE77954. Methylation values were reported as M-values (log 2 ratios of methylated to unmethylated probes). To determine probe-wise methylation scores, we associated each probe with the nearest annotated transcriptional start site, focusing on probes within the putative promoter regions of annotated genes (Supplementary Table S6).

To subgroup TCGA tumors into MLH1 methylation high/low categories, we obtained raw Illumina 450K Beadchip microarray data for 292 tumor samples from the TCGA and preprocessed it as described above. As described previously, we examined probe clusters and selected the CpG island of the bidirectional MLH1/EP302AP1 promoter. The normalized methylation scores (M-value) were transformed to z-scores by centering on the mean and scaling to unit variance. As expected, visual inspection revealed a strongly bimodal distribution and samples with positive scores were assigned the MLH1/CIMP+ subgroup label.
Quantitative methylation-specific PCR
To quantify the percentage of methylated EREG at probe cg.19308222 in the validation sample set, CRC cell lines and in trial patient samples, quantitative methylation-specific PCR assays targeting either a fully methylated EREG (EREG qMSP), or a fully unmethylated EREG (EREG qUSP) at this specific position were designed. The sequences of real-time PCR primers for EREG quantitative methylation-specific PCR are as follows: EREG qMSP, forward, 5′-AGGGTTTATAGAAGAAGG-3′; reverse, 5′-TATCAATAATTGACCCTCTT-3′ and probes, 5′-TATCAATAATTGACCCTCTT-3′ and probe, 5′-TATCAATAATTGACCCTCTT-3′; reverse, 5′-TATCAATAATTGACCCTCTT-3′ and probe, 5′-TATCAATAATTGACCCTCTT-3′; forward, 5′-TAGGGTTTATAGAAGAAAG-3′; reverse, 5′-TATCAATAATTGACCCTCTT-3′; and probe, 5′-TATCAATAATTGACCCTCTT-3′.

5-Aza-dC treatment
All cell lines were obtained from the Genentech cell lines repository and were authenticated by STR profiling and confirmed to be free of mycoplasma contamination. Cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 2 mM l-glutamine. Cells were seeded on day 0 at 4000–9000 cells per cm² and dosed with 500 nM 5-aza-2′-deoxycytidine (5-Aza-dC) (cat. no.: A3656; Sigma-Aldrich, St Louis, MO, USA) or dimethyl sulfoxide (DMSO) control on days 1 and 3. On day 5, cells were harvested and lysed for quantitative methylation-specific PCR assays targeting either a fully methylated DNA from EpiTect (Qiagen).

Statistical analysis
To evaluate the correlation between normalized mRNA expression and mean promoter methylation (M-value) during CRC progression, Spearman’s rank-correlation coefficients were calculated for all genes that were (1) assayed on the gene expression arrays and (2) whose promoter(s) contained one or more probes assayed on the methylation arrays. In the case of genes for which methylation probes mapped to multiple alternative promoters, the promoter displaying the highest interquartile range across the full data set was selected. To evaluate the differential expression of annotated EGFR and VEGFR signaling pathway components during CRC progression, an unpaired t-test was used and two-sided P-value was derived. The area of interest was reviewed at x20 magnification. RNAscope signal was binned into five groups based on the number of dots per cell as follows: bin 0 = 0 dots per cell, bin 1 = 1–3 dots per cell, bin 2 = 4–9 dots per cell, bin 3 = 10–15 dots per cell and bin 4 = > 15 dots per cell with > 10% of dots in clusters. Each sample was evaluated for the percentage of cells in each bin. The H-score was calculated as follows, H-score = (0 \times (% of cells in bin 0)) + (1 \times (% of cells in bin 1)) + (2 \times (% of cells in bin 2)) + (3 \times (% of cells in bin 3)) + (4 \times (% of cells in bin 4)), with an H-score scale of 0–400. Bright field images were acquired using a Zeiss Axio Imager M1 microscope (Zeiss, Oberkochen, Germany) using a ×40 objective.

CONFLICT OF INTEREST
XQ, TS, LF, KW, KO, SL, YW, RB, EP, AP, LA, MRL, GMH and OK are current or former employees and stock holders of Genentech/Roche. The authors declare no conflict of interest.

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