ORIGINAL ARTICLE
Distinct gene expression profiles of proximal and distal colorectal cancer: implications for cytotoxic and targeted therapy

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Colorectal cancer (CRC) is a heterogeneous disease with genetic profiles and clinical outcomes dependent on the anatomic location of the primary tumor. How location has an impact on the molecular makeup of a tumor and how prognostic and predictive biomarkers differ between proximal versus distal colon cancers is not well established. We investigated the associations between tumor location, KRAS and BRAF mutation status, and the messenger RNA (mRNA) expression of proteins involved in major signaling pathways, including tumor growth (epidermal growth factor receptor (EGFR)), angiogenesis (vascular endothelial growth factor receptor 2 (VEGFR2)), DNA repair (excision repair cross complement group 1 (ERCC1)) and fluoropyrimidine metabolism (thymidylate synthase (TS)). Formalin-fixed paraffin-embedded tumor specimens from 431 advanced CRC patients were analyzed. The presence of seven different KRAS base substitutions and the BRAF V600E mutation was determined, ERCC1, TS, EGFR and VEGFR2 mRNA expression levels were detected by reverse transcriptase-PCR. BRAF mutations were significantly more common in the proximal colon (P < 0.001), whereas KRAS mutations occurred at similar frequencies throughout the colorectum. Rectal cancers had significantly higher ERCC1 and VEGFR2 mRNA levels compared with distal and proximal colon tumors (P = 0.001), and increased TS levels compared with distal colon cancers (P = 0.02). Mutant KRAS status was associated with lower ERCC1, TS, EGFR and VEGFR2 gene expression in multivariate analysis. In a subgroup analysis, this association remained significant for all genes in the proximal colon and for VEGFR2 expression in rectal cancers. The mRNA expression patterns of predictive and prognostic biomarkers, as well as associations with KRAS and BRAF mutation status depend on primary tumor location. Prospective studies are warranted to confirm these findings and determine the underlying mechanisms.

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INTRODUCTION
The translation of clinically relevant biomarkers into personalized medicine for colorectal cancer (CRC) patients has been proven a challenging endeavor. For instance, although RAS mutant status predicts lack of response towards epidermal growth factor receptor (EGFR)-directed antibodies,¹–⁴ many patients with RAS wild-type tumors do not benefit from such therapy.⁵–⁷ Similarly, the predictive utility of EGFR⁸–¹⁰ expression and the V600E BRAF mutation¹¹,¹² has been limited, and studies evaluating the predictive value of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2; Zhang et al.¹³ and Schimanski et al.¹⁴) expression for VEGF-targeted drugs have yielded inconsistent results. Likewise, molecular determinants of response towards cytotoxic agents, including fluoropyrimidines (that is, thymidylate synthase (TS)) and platinum agents (that is, excision repair cross complement group 1 (ERCC1)), have been retrospectively associated with response rates and survival¹⁵–¹⁸ but are not yet prospectively validated. Limitations impeding biomarker development include methodological differences across studies, redundancy in signaling, as well as tumor heterogeneity.

Recent data suggests that the location of a colorectal tumor (that is proximal vs distal vs rectum) may have an impact on its molecular landscape¹⁹,²⁰ and clinical behavior.²¹,²² Microarray DNA analyses have revealed over 1000 genes with different expression patterns between ascending and descending colon cancers,²³ which partly reflect the distinct embryonic origin (that is, midgut vs hindgut) and vascular supply (that is, superior vs inferior mesenteric artery) of the proximal and distal colon.²⁴ Phenotypically, proximal tumors are prone to microsatellite instability (MSI),²⁵,²⁶ BRAF mutations²⁷,²⁸ and poorly differentiated histology,²⁹,³⁰ whereas distal tumors are characterized by loss of heterozygosity and TP53 mutations.²⁹,³⁰ Clinically, proximal tumors tend to present at later stages³¹ and are associated with worse overall survival³² relative to their distal counterparts. Although the presence of anatomic-based CRC gene signatures has been established, associations between predictive and prognostic biomarker expression and tumor location are not well understood. Such knowledge may shed insight on interactions linking tumor location and treatment response and outcomes, which may guide personalized therapy in the future. On this

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premise, we used a commercially available database to determine the relationship between primary tumor site and the expression of biomarkers involved in major signaling pathways in advanced CRC patients. Specifically, we examined the associations between tumor location and gene expression levels of proteins involved in tumor growth (EGFR), angiogenesis (VEGFR2), DNA repair (ERCC1) and chemotherapy drug metabolism (TS), as well as KRAS and BRAF mutation status.

MATERIALS AND METHODS

Study design and patient population

We conducted a retrospective analysis of data collected from a cohort of 578 patients with stage IV CRC, whose tumor tissue was submitted to Response Genetics (RGI, Los Angeles, CA, USA), a Clinical Laboratory Improvement Amendments-certified and College of American Pathologists-accredited laboratory, for comprehensive molecular testing (ColonDX; Response Genetics Inc., Los Angeles, CA, USA). Patient samples were submitted from both private and academic healthcare institutions across the United States between 2007 and 2010. Formalin-fixed paraffin embedded tumor specimens were tested for KRAS and BRAF mutation status, as well as messenger RNA (mRNA) expression levels of ERCC1, TS, EGFR and VEGFR2. Only patients whose specimens had sufficient tissue for analysis of at least one gene of interest (that is, ERCC1, TS, EGFR and VEGFR2) and detection of either KRAS and/or BRAF mutations, as well as data regarding tumor location, were included in the study. Tumor samples from metastatic sites, in which the primary tumor location was unknown, were excluded. A total of 431 patients were included in the final analysis.

Information regarding primary tumor location, patient age and gender, tumor grade and histology were extracted from pathology reports submitted with the tissue specimens and recorded by two of the authors (MMK and DLH). Specifically, the splenic flexure was used to distinguish proximal from distal tumors. Tumors within 15 cm of the anal verge were designated as originating in the rectum.

Tumor tissue preparation and gene expression analysis

Tumor tissue from study patients was obtained at the time of diagnosis before surgery and at the time of surgical resection. Hematoxylin and eosin-stained sections of all formalin-fixed paraffin embedded specimens were evaluated by a board-certified pathologist for tumor content. Formalin-fixed paraffin-embedded tissues were dissected. Ten-micrometer thick slides were obtained from the identified areas with the highest tumor concentration and were mounted on uncoated glass slides. For histologic diagnosis, three sections representative of the beginning, middle and end of the tissue were stained with hematoxylin and eosin, using the standard method. Before microdissection, sections were de-paraffinized in xylene for 10 min, hydrated with 100%, 95% and 70% ethanol, and then washed in H2O for 30 s. Following microdissection of tumor cells, the sections were stained with nuclear fast red (American Master Tech Scientific, Lodi, CA, USA) for 20 s and rinsed in water for 30 s. Samples were then dehydrated with 70%, 95% and 100% ethanol for 30 s each, followed by xylene for 10 min. The slides were then completely air-dried. Laser capture microdissection (PALM Microlaser Technologies AG, Bernried, Germany) was carried out in all tumor samples to ensure that only tumor cells were dissected.33 The dissected particles of tissue were transferred to a reaction tube containing 400 μl of RNA buffer for lysis of tumor cells.

After lysis of the tumor cells, RNA and DNA were isolated separately from the specimen. RNA isolation from paraffin-embedded samples was done according to a proprietary procedure defined by RGI (US Patent 6248535). The RNA was then reverse-transcribed to cDNA as described previously.34 DNA was either directly extracted or back extracted from the organic phase, both with an RGI-patented method (US Patent 6248535).

Quantitation of gene mRNA expression levels of ERCC1, TS, EGFR, VEGFR2 and an internal reference (β-actin) cDNA was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System (TaqMan); Perkin-Elmer Applied Biosystems, Waltham, MA, USA) as previously described.35 Isolated RNA was reverse-transcribed to cDNA, followed by reverse transcriptase-PCR using specific primers and probes. The PCR reaction mixture consisted of the following: 1200 nmol l−1 of each primer; a 200 nmol l−1 probe; 0.4 μl of AmpliTag Gold Polymerase; 200 mmol l−1 of dATP, dCTP, dGTP, dTTP; 3.5 mmol l−1 Mg2+; and 1 × TaqMan Buffer A containing a reference dye added to a final volume of 20 μl (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min.

The ERCC1 primers and probe sequences used were as follows: forward primer, 5′-GGGAAATTTGCGGCGATTAATGC-3′; reverse primer, 5′-GGCGGAGTGCAAGCAGAAG-3′; probe, 6FAM-5′-CAACAGGTCCTGGGTCTC-3′. The TS primers and probe sequences used were as follows: forward primer, 5′-TGCGCTCTGCTGCCGGAAT-3′; reverse primer, 5′-GGTACCCAGAGGAACGTC-3′. The VEGFR2 primers and probe sequences used were as follows: forward primer, 5′-CCGTGTG TCTCTGCTGGA-3′; reverse primer, 5′-CTAAGCTTGGGCAATAGAG-3′; probe, 6FAM-5′-ACATCGAGCAACCAAGGCGGC-3′. The β-actin primers and probe sequences used were as follows: forward primer, 5′-GAGGGGCTTCAGACCT-3′; reverse primer, 5′-CTCCTATATTGCTACCCAGA-3′; probe, 6FAM-5′-ACACACCGGCGGAGG-3′.

For each sample, parallel TaqMan PCR reactions were carried out for each gene of interest and the β-actin reference gene to normalize for input cDNA. Results were obtained as a ratio of the PCR fluorescent signals of each gene of interest relative to the reference gene, β-actin.

KRAS mutation analysis was performed with a RGI-designed mutation assay by reverse transcriptase-PCR using specifically designed primers and probes to detect each of the following mutations: Gly12Ala (GGT→GCT) 522; Gly12Asp (GGT→GAT) 521; Gly12Arg (GGT→GTA) 518; Gly12Cys (GGT→TGT) 516; Gly12Ser (GGT→AGT) 517; Gly12Val (GGT→GTT) 520; Gly13Asp (GGC→GAC) 532. After reverse transcriptase-PCR, all collected data were analyzed through an RGI Excel template, and according to delta-CT values the mutation status was established. BRAF V600E mutations were detected by dye terminator sequencing.

Statistical analysis

mRNA expression levels of ERCC1, TS, EGFR and VEGFR2 were summarized and analyzed with Wilcoxon two-sample tests to detect differences based on KRAS and BRAF mutation status within each tumor site. Pairwise differences between the expression of the four examined genes across tumor sites, independent of KRAS and BRAF mutation status, were then determined by Wilcoxon two-sample tests, with significance determined by Kruskal–Wallis testing. Bonferroni method was used to correct P-value for multiple comparisons. All values were reported as medians and ranges, with a significance P-value cutoff of 0.05. Analyses were performed using Statistical Analysis Software (SAS) version 9.3 (SAS Institute, Cary, NC, USA).

RESULTS

Patient and tumor characteristics

Among 431 patients with advanced CRC, 58% were men and 42% were women, with a median age of 61 years (range 27–92 years old) (Table 1). The distribution of tumor site was as follows: 39% distal, 40% proximal and 21% rectal. The frequency of KRAS and BRAF mutations among all patients was 42% and 8%, respectively. BRAF mutations were significantly more common in the proximal colon (14%), followed by distal colon (5%) and rectal tumors (2%) (P < 0.001), whereas KRAS mutations occurred at similar frequencies throughout the colorectum (P = 0.51) (Table 1).

ERCC1 expression by tumor location and KRAS, BRAF mutation status

Rectal cancers demonstrated higher ERCC1 expression (median 1.11 (0.23–4.23)) than either distal (median 0.83 (0.17–6.66)) or proximal colon tumors (median 0.91 (0.26–3.06); P = 0.001; Figure 1a and Table 2). Among all patients, KRAS mutant status was associated with decreased ERCC1 expression (median 0.87 (0.23–2.96)) compared with KRAS wild-type status (median 0.97 (0.17–6.66); P = 0.01; Table 3). In subgroup analysis, this trend remained significant in proximal tumors (KRAS mutant median 0.82 (0.26–2.95); KRAS wild-type median 0.98 (0.28–3.06); P = 0.005; Table 2). A similar
other differences detected in TS expression between tumor sites. mRNA levels (median 2.08 (0.52–2.12)) compared with BRAF wild-type tumors (median 1.75 (0.33–7.17)). There was no significant association between BRAF mutation status and TS expression (Table 3) in the subgroup of proximal tumors (n = 156), there was a non-significant trend toward increased TS expression in BRAF mutant tumors (median 0.96 (0.28–2.47)) compared with BRAF wild-type tumors (median 0.88 (0.26–3.06); P = 0.06).

TS expression by tumor location and KRAS, BRAF mutation status
Rectal tumors had significantly higher TS expression levels (median 2.60 (0.77–21.43)) than distal colon cancers (median 2.12 (0.65–10.26); P = 0.02; Figure 1b and Table 2). There were no other differences detected in TS expression between tumor sites. Patients with KRAS mutant tumors had significantly lower TS mRNA levels (median 2.08 (0.52–21.43)) than those with KRAS wild-type cancers (median 2.38 (0.65–18.99); P = 0.03; Table 3). When stratified by tumor location, this association remained significant only in proximal colon tumors (KRAS mutant median 1.90 (0.52–9.43); KRAS wild-type median 2.38 (0.65–13.81); P = 0.03; Table 2).

The BRAF V600E mutation was predictive of increased TS expression levels (BRAF mutant median 3.38 (1.01–13.81); BRAF wild-type median 2.17 (0.52–21.43); P < 0.001) among all patients (Table 3). This association remained significant in proximal colon tumors (BRAF mutant median 3.68 (1.01–13.81); BRAF wild-type median 2.07 (0.52–11.10); P = 0.003; Figure 2a).

EGFR expression by tumor location and KRAS, BRAF mutation status
EGFR expression was similar across all tumor sites (P = 0.20; Figure 1c and Table 2). However, EGFR expression was decreased in KRAS-mutated cancers (median 1.61 (0.33–7.17)) compared with KRAS wild-type cancers (median 2.06 (0.63–7.28); P < 0.001) among all patients (Table 3). In subgroup analysis, this difference remained significant in proximal colon cancers (KRAS mutant median 1.60 (0.33–7.17); KRAS wild-type median 2.12 (0.63–7.45); P < 0.001; Table 2).

BRAF mutant status was associated with increased EGFR levels across all patients (BRAF mutant median 2.20 (1.12–3.50); BRAF wild-type median 1.75 (0.33–7.18); P = 0.002; Table 3). This association remained significant in the proximal colon cohort (BRAF mutant median 2.23 (1.45–3.50); BRAF wild-type median 1.76 (0.33–7.45); P = 0.002; Figure 2b).

VEGFR2 expression by tumor location and KRAS, BRAF Mutation Status
Rectal tumors demonstrated significantly higher VEGFR2 expression (1.77 (0.23–13.00)) than distal (1.24 (0.18–7.66)) and proximal (1.10 (0.24–6.10)) tumors (P < 0.001 for both comparisons; Figure 1d and Table 2). Furthermore, VEGFR2 mRNA levels were significantly lower in mutated KRAS tumors (median 1.19 (0.23–7.66)) than in wild-type tumors (median 1.35 (0.18–13.00); P = 0.003; Table 3). In subgroup Table 1. Colorectal cancer patient and tumor characteristics (N = 431)

| Characteristic                        | Distal colon (N = 170) | Proximal colon (N = 171) | Rectum (N = 90) | P-valueb |
|--------------------------------------|------------------------|--------------------------|----------------|----------|
| Age in years: median (range)         | 59 (27–92)             | 66 (29–89)               | 59 (31–85)     | < 0.001  |
| Gender                               | 97 (57)                | 92 (54)                  | 61 (68)        | 0.09     |
| Men                                  | 73 (43)                | 79 (46)                  | 29 (32)        |          |
| Tumor grade                          |                        |                          |                |          |
| Well differentiated                  | 7 (4)                  | 10 (6)                   | 8 (9)          | 0.04     |
| Moderately differentiated            | 106 (62)               | 88 (51)                  | 52 (58)        |          |
| Poorly differentiated                | 31 (18)                | 48 (28)                  | 14 (16)        |          |
| Unknown                              | 26 (15)                | 25 (15)                  | 16 (18)        |          |
| KRAS mutation status                 |                        |                          |                | 0.51     |
| Mutant                               | 69 (41)                | 78 (46)                  | 33 (37)        |          |
| Wild type                            | 91 (54)                | 83 (49)                  | 46 (51)        |          |
| Unknown                              | 10 (6)                 | 10 (6)                   | 11 (12)        |          |
| KRAS mutation                        |                        |                          |                | 0.012    |
| Gly12Ala                             | 8 (12)                 | 1 (1)                    | 2 (6)          |          |
| Gly12Asp                             | 20 (29)                | 31 (40)                  | 9 (27)         |          |
| Gly12Arg                             | 2 (3)                  | 0 (0)                    | 1 (3)          |          |
| Gly12Cys                             | 9 (13)                 | 5 (6)                    | 3 (9)          |          |
| Gly12Ser                             | 6 (9)                  | 5 (6)                    | 3 (9)          |          |
| Gly12Val                             | 20 (29)                | 15 (19)                  | 5 (15)         |          |
| Gly13Asp                             | 4 (6)                  | 21 (27)                  | 10 (30)        |          |
| BRAF mutation status                 |                        |                          |                | < 0.001  |
| Mutant                               | 9 (5)                  | 24 (14)                  | 2 (2)          |          |
| Wild type                            | 147 (86)               | 133 (78)                 | 81 (90)        |          |
| Unknown                              | 14 (8)                 | 14 (8)                   | 7 (8)          |          |

*Proximal and distal colon tumors were demarcated by the splenic flexure. Rectal tumors were defined as those within 15 cm of the anal verge. **P-value was based on Kruskal–Wallis Test for age and χ²-test for other characteristics. Patients with unknown characteristics were excluded. Bolded P values were statistically significant.
analysis, this relationship persisted in proximal (KRAS mutant median 0.91 (0.24–6.10); KRAS wild-type median 1.29 (0.33–5.94); \( P = 0.01 \)) and rectal (KRAS mutant median 1.53 (0.23–3.62); KRAS wild-type median 2.21 (0.37–13.00); \( P = 0.02 \)) cancers (Table 2).

There was no significant association between BRAF mutation status and VEGFR2 expression (Table 3). In subgroup analysis, proximal tumors \((n = 106)\) with the BRAF V600E mutation trended toward increased VEGFR2 expression (median 1.48 (0.56–2.63)) compared with BRAF wild-type tumors (median 1.06 (0.24–6.10); \( P = 0.07 \)).

**DISCUSSION**

CRC subsites are characterized by distinct genetic and histopathological features, but the association between tumor location and prognostic and predictive biomarker expression is not well delineated. We evaluated whether primary tumor site influences KRAS and BRAF mutation status, and the mRNA expression of biomarkers reflecting DNA repair, fluoropyrimidine metabolism, tumor cell growth and angiogenesis in advanced CRC. Our analysis revealed that each tumor site has a unique molecular phenotype, which may predict chemotherapeutic and antibody drug sensitivity as well as clinical outcomes (Figure 3). Furthermore, we found that KRAS and BRAF mutation status is associated with biomarker gene expression, and these relationships depend on tumor location.

**KRAS and BRAF mutation status by tumor location**

The distribution of KRAS mutations was similar across tumor sites. Although others\(^{36–38}\) have demonstrated more frequent KRAS mutations in the proximal colon, the data are not consistent,\(^{39}\) and this may reflect methodologic variation as well as different KRAS mutations being examined between studies. Conversely, BRAF V600E mutations were more common in proximal cancers in our cohort, which is consistent with prior data.\(^{12,28,37}\) The predilection of BRAF mutations for the proximal colon partly reflects increased MSI in this region,\(^{26,40,41}\) as microsatellite unstable tumors are more enriched with BRAF mutations.\(^{42,43}\)
Cancers had significantly increased TS levels compared with distal colon cancers. Our group compared with distal and proximal colon tumors, in addition to (that is, enzymatic vs nuclear or cytoplasmic protein expression) The Pharmacogenomics Journal (2015), 354–362 therapy between rectal and colonic tumors,28,37 ours is the first study to reveal anatomic-based differences in ERCC1 and VEGFR2 expression. Irrespective of KRAS or BRAF mutation status, colorectal tumors have previously shown contrasting TS expression by tumor location and VEGFR2 expression depends on tumor location. Although prior investigations have demonstrated divergent patterns of gene expression, metastatic spread and response to therapy between rectal and colonic tumors,28,37 ours is the first study to reveal anatomic-based differences in ERCC1 and VEGFR2 expression. Irrespective of KRAS or BRAF mutation status, colorectal cancers had significantly higher ERCC1 and VEGFR2 mRNA levels compared with distal and proximal colon tumors, in addition to increased TS levels compared with distal colon cancers. Our group and others have previously shown contrasting TS expression by CRC subsite,44–46 although the different assays used (that is, enzymatic vs nuclear or cytoplasmic protein expression) confounds interpretation. In sum, our findings suggest that the efficacy of oxaliplatin, anti-angiogenic and fluoropyrimidine agents may differ between proximal and distal tumors, and this hypothesis warrants prospective validation in the clinical trial setting. Furthermore, stratification by specific anatomic site (rather than broad categories of proximal vs distal tumors) may offer more useful predictive and prognostic information.37

Table 2. ERCC1, TS, EGFR, VEGFR2 Gene Expression Levels by Tumor Site and KRAS Mutation Status

| Gene   | KRAS mutation status | Primary tumor location |       |  |       |  |       |  |
|--------|---------------------|------------------------|-------|---|-------|---|-------|---|
|        |                     | Distal colon           | Proximal colon | Rectum |       |  |       |  |
|        | N | Median (range) | N | Median (range) | N | Median (range) |  |       |  |
| ERCC1  | Mutant | 67 | 0.79 (0.27–2.96) | 77 | 0.82 (0.26–2.95) | 32 | 0.96 (0.23–2.60) |  |       |  |
|        | Wild type | 86 | 0.84 (0.17–6.66) | 79 | 0.97 (0.28–3.06) | 46 | 1.23 (0.37–4.23) |  |       |  |
|        | All | 162 | 0.83 (0.17–6.66) | 162 | 0.91 (0.26–3.06) | 88 | 1.11 (0.23–4.23) |  |       |  |
|        | PairwiseP | 0.80 |  |  |  | 0.005 |  |  | 0.001 |  |       |  |

TS Mutant | 67 | 1.92 (0.81–9.26) | 76 | 1.90 (0.52–9.43) | 31 | 2.69 (0.77–21.43) |  |       |  |
| Wild type | 84 | 2.25 (0.65–10.02) | 79 | 2.38 (0.65–13.81) | 46 | 2.66 (0.97–18.99) |  |       |  |
| All | 160 | 2.12 (0.65–10.26) | 161 | 2.18 (0.52–13.81) | 85 | 2.60 (0.77–21.43) |  |       |  |
| PairwiseP | 0.03 |  |  |  | 0.00 |  |  | 0.02 |  |       |  |

EGFR Mutant | 67 | 1.60 (0.79–5.24) | 76 | 1.60 (0.33–7.17) | 32 | 1.66 (0.78–4.08) |  |       |  |
| Wild type | 88 | 1.78 (0.63–5.68) | 76 | 2.12 (0.63–7.45) | 44 | 2.20 (0.80–71.28) |  |       |  |
| All | 164 | 1.70 (0.60–5.68) | 161 | 1.88 (0.33–7.45) | 83 | 1.92 (0.75–71.28) |  |       |  |
| PairwiseP | 0.20 |  |  |  | 0.01 |  |  | 0.02 |  |       |  |

VEGFR2 Mutant | 42 | 1.32 (0.25–7.66) | 50 | 0.91 (0.24–6.10) | 25 | 1.53 (0.23–3.62) |  |       |  |
| Wild type | 66 | 1.21 (0.18–6.64) | 54 | 1.29 (0.33–5.94) | 38 | 2.21 (0.37–13.00) |  |       |  |
| All | 111 | 1.23 (0.18–7.66) | 108 | 1.10 (0.24–6.10) | 68 | 1.77 (0.23–13.00) |  |       |  |
| PairwiseP | <0.001 |  |  |  | 0.01 |  |  | 0.02 |  |       |  |

*Based on Wilcoxon two-sample test for differences by KRAS mutation status in each tumor site. *Based on Kruskal–Wallis test for differences across tumor sites. *Based on Wilcoxon two-sample test for pairwise differences by two tumor sites adjusting for multiple comparisons; includes patients with unknown KRAS mutation status. The same symbol represents a significant pairwise difference. Bolded P values were statistically significant.

Table 3. ERCC1, TS, EGFR, VEGFR2 Gene Expression Levels by KRAS and BRAF Mutation Status

| Gene   | KRAS mutation status |       |  |  |  |  |
|--------|---------------------|-------|---|---|---|---|
|        | Mutant | N | Median (range) | Wildtype | N | Median (range) | Mutant | N | Median (range) | Wildtype | N | Median (range) |  |       |  |
| ERCC1  | P value* | 0.01 | 211 | 0.98 (0.17–6.66) | 35 | 0.94 (0.28–2.47) | 356 | 0.91 (0.20–6.66) |  |       |  |
|        | P value* | 0.03 | 209 | 2.38 (0.65–18.99) | 34 | 3.38 (1.01–13.81) | 352 | 2.17 (0.52–21.43) |  |       |  |
| EGFR P value* | <0.001 | 208 | 2.06 (0.63–71.28) | 33 | 2.20 (1.12–3.50) | 349 | 1.75 (0.33–71.28) |  |       |  |
| VEGFR2 P value* | <0.001 | 158 | 1.35 (0.18–13.00) | 26 | 1.48 (0.29–3.41) | 252 | 1.25 (0.18–13.0) |  |       |  |

Based on Kruskal-Wallis test.

ERCC1, TS, EGFR and VEGFR2 expression by tumor location

Although prior investigations have demonstrated divergent patterns of gene expression, metastatic spread and response to therapy between rectal and colonic tumors,28,37 ours is the first study to reveal anatomic-based differences in ERCC1 and VEGFR2 expression. Irrespective of KRAS or BRAF mutation status, colorectal cancers had significantly higher ERCC1 and VEGFR2 mRNA levels compared with distal and proximal colon tumors, in addition to increased TS levels compared with distal colon cancers. Our group and others have previously shown contrasting TS expression patterns by CRC subsite,44–46 although the different assays used (that is, enzymatic vs nuclear or cytoplasmic protein expression) confounds interpretation. In sum, our findings suggest that the efficacy of oxaliplatin, anti-angiogenic and fluoropyrimidine agents may differ between proximal and distal tumors, and this hypothesis warrants prospective validation in the clinical trial setting. Furthermore, stratification by specific anatomic site (rather than broad categories of proximal vs distal tumors) may offer more useful predictive and prognostic information.37

Influence of KRAS and BRAF mutation status on ERCC1, TS, EGFR and VEGFR2 expression depends on tumor location

Mutant KRAS status was associated with lower expression of ERCC1, TS, EGFR and VEGFR2 among all patients. In multivariate
This association remained significant for all biomarkers in the proximal colon. In addition, among rectal cancers, KRAS mutant tumors had decreased VEGFR2 expression than KRAS wild-type ones. The fact that the association between KRAS status and biomarker gene expression varied by tumor location supports distinct carcinogenic mechanisms across tumor sites. It also suggests the presence of a heterogeneous intestinal microenvironment, and that tumor–stromal interactions and epigenetic modifications are critical in mediating the effects of cytotoxic and targeted agents.

One such epigenetic association may exist between the KRAS and nucleotide excision repair pathways, particularly in proximal colon tumors. In vitro studies in COLO320DM colon cancer cell lines have demonstrated that KRAS suppression by small interfering RNAs leads to ERCC1 overexpression and oxaliplatin resistance, whereas KRAS activation may decrease ERCC1 gene expression through upregulation of DNA methyltransferase 3β and subsequent ERCC1 hypermethylation, promoting oxaliplatin sensitivity. It follows that one anatomic-based link between KRAS and nucleotide excision repair may lie in methylation differences between tumor sites. The CpG island methylator phenotype is more common in proximal and KRAS-mutant tumors. Our findings may also help explain clinical outcome data from the PRIME and OPUS trials. Patients from these studies with KRAS mutant tumors, who were treated with FOLFOX alone showed a trend toward improved progression-free survival, relative to those with KRAS wild-type tumors. Such a relationship was not observed in the CRYSTAL trial, which used irinotecan-based chemotherapy. Identifying the regulatory mechanisms between KRAS activation, nucleotide excision repair and tumor location may offer novel and more personalized drug targets in future investigations.

TS expression was also influenced by KRAS and BRAF mutation status in a location-dependent manner. The lower TS mRNA levels in KRAS-mutated proximal colon tumors suggest increased fluoropyrimidine sensitivity in this cohort. Previous studies have not shown a significant association between KRAS mutation status and response to fluoropyrimidines or TS expression regardless of tumor location, although these studies employed enzymatic rather than mRNA assays, which limits direct comparison with our results. In contrast, the BRAF V600E mutation was associated with increased TS expression among proximal cancers. This may provide a potential explanation for the inferior outcomes seen in patients with BRAF-mutated tumors treated with 5-fluorouracil-based regimens. Both CpG island methylator phenotype and MSI status have also been independently linked with lack of clinical benefit from 5-fluorouracil, although examinations of the predictive utility of CpG island methylator phenotype and MSI and the correlation between MSI and TS expression have yielded conflicting data. As such, further studies are needed to better define the relationship between methylation patterns, mismatch repair and TS expression in proximal tumors.

Among proximal colon cancers, KRAS-mutated tumors had significantly decreased EGFR and VEGFR2 levels, compared with wild-type tumors. These findings suggest that in addition to constitutive KRAS activation, downregulation of EGFR and angiogenic pathways may provide another reason for diminished response toward targeted antibodies in these patients. Indeed, in a recent subgroup analysis of the FIRE-3 trial, patients with proximal tumors benefited less from anti-EGFR-directed therapies than those with distal cancers. It also suggests that the EGFR and

![Figure 2](image-url) (a) Thymidylate synthase (TS) expression by BRAF mutation status in proximal colon cancers. (b) Epidermal growth factor receptor (EGFR) expression by BRAF mutation status in proximal colon cancers.

![Figure 3](image-url) Gene expression profiles by primary tumor location.
VEGF signaling pathways share regulatory pathways, including the mitogen-activated protein kinase/phosphoinositide 3-kinase, signal transducer and activator of transcription and hypoxia-inducible factor signaling cascades, as demonstrated in cell line and xenograft models. Within the rectal cancer cohort, KRAS mutant tumors had decreased VEGFR2 expression compared with the KRAS wild-type group. This is consistent with clinical data showing significantly improved pathologic complete response rates among KRAS wild-type rectal cancer patients receiving cetuximab-based neoadjuvant chemoradiation, and whose tumors had increased intratumoral VEGFR2 expression.

In contrast to KRAS mutant tumors, BRAF-mutated cancers had increased EGFR expression in the proximal colon subgroup. As our samples came from untreated patients, this supports the hypothesis that EGFR overexpression may be an inherent rather than acquired resistance mechanism toward BRAF inhibitors in CRC patients.

Our study has its limitations, the first of which is its retrospective design. We could not account for potential confounding variables, including history of prior cancers, patient ethnicity and MSI status, any of which could have independently influenced biomarker expression. An extended Ras analysis examining mutations outside of exon 2 may have yielded additional associations between tumor location and gene expression. Furthermore, information regarding prior treatment, particularly in patients who may have had liver-limited metastases and received chemotherapy before surgical resection is not known. In addition, we used the splenic flexure as the dividing line between proximal and distal sided tumors, as the precise location of tumors within the transverse colon (that is, proximal two-thirds vs distal one-third) was not always documented, and this does not reflect boundaries based on blood supply and embryonic origin. Importantly, the lack of outcome data precludes definitive conclusions about the prognostic significance of the demonstrated anatomic-based associations in gene expression.

CONCLUSIONS
CRC comprises a spectrum of tumors with unique carcinogenic mechanisms, stromal interactions and clinical outcomes based on primary site. Our study is the first to demonstrate that the mRNA expression of predictive and prognostic biomarkers and their relationship with KRAS and BRAF mutation status are contingent on anatomic location. Our analysis further emphasizes the distinct biology among CRCs and that tumor location should be included in clinical decision-making. Prospective studies ought to confirm our findings and incorporate the role of tumor site in understanding CRC molecular heterogeneity and evolving phenotypes with treatment. A more refined use of biomarkers should advance clinical trial design, drug development and patient outcomes.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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