A let-7 microRNA-Binding Site Polymorphism in KRAS Predicts Improved Outcome in Patients with Metastatic Colorectal Cancer Treated with Salvage Cetuximab/Panitumumab Monotherapy

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Abstract

Purpose: An inherited mutation in KRAS (LCS6-variant or rs61764370) results in altered control of the KRAS oncogene. We studied this biomarker’s correlation to anti-EGFR monoclonal antibody (mAb) therapy response in patients with metastatic colorectal cancer.

Experimental Design: LCS6-variant and KRAS/BRAF mutational status was determined in 512 patients with metastatic colorectal cancer treated with salvage anti-EGFR mAb therapy, and findings correlated with outcome. Reporters were tested in colon cancer cell lines to evaluate the differential response of the LCS6-variant allele to therapy exposure.

Results: In this study, 21.2% (109 of 512) of patients with metastatic colorectal cancer had the LCS6-variant (TG/GG), which was found twice as frequently in the BRAF-mutated versus the wild-type (WT) group (P = 0.03). LCS6-variant patients had significantly longer progression-free survival (PFS) with anti-EGFR mAb monotherapy treatment in the whole cohort (16.85 vs. 7.85 weeks; P = 0.019) and in the double WT (KRAS and BRAF) patient population (18 vs. 10.4 weeks; P = 0.039). Combination therapy (mAbs plus chemotherapy) led to improved PFS and overall survival (OS) for nonvariant patients, and brought their outcome to levels comparable with LCS6-variant patients receiving anti-EGFR mAb monotherapy. Combination therapy did not lead to improved PFS or OS for LCS6-variant patients. Cell line studies confirmed a unique response of the LCS6-variant allele to both anti-EGFR mAb monotherapy and chemotherapy.

Conclusions: LCS6-variant patients with metastatic colorectal cancer have an excellent response to anti-EGFR mAb monotherapy, without any benefit from the addition of chemotherapy. These findings further confirm the importance of this mutation as a biomarker of anti-EGFR mAb response in patients with metastatic colorectal cancer, and warrant further prospective confirmation.

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Introduction

In the western world, colorectal cancer remains a major public health problem, with an estimated 136,830 new cases and 50,310 deaths estimated to occur in 2014 in the United States alone (1). The incorporation of two mAbs targeting EGFR (anti-EGFR mAbs), cetuximab and panitumumab, in metastatic colorectal cancer clinical practice either given as monotherapy or in combination with chemotherapy, has proved to provide a modest clinical benefit in pretreated patients (2–5). Nevertheless, their efficacy is restricted to a subset of patients, as nonrandomized retrospective studies (6–9), retrospective analysis of prospective randomized trials (10–13), a summary of the above mentioned publications, and a grand European consortium study (14) have demonstrated. For example, the presence of tumor-acquired KRAS mutations are predictive of resistance to anti-EGFR mAb therapy and are associated with worse prognosis and shorter survival.
Translational Relevance

The KRAS-variant (LCS6-variant or rs61764370) represents a new type of germ-line mutation, which is located in the nonprotein coding region (the 3’-untranslated region), of the KRAS oncogene. This mutation disrupts binding of the let-7 miRNA to KRAS and leads to KRAS and downstream cellular pathway signaling disruption, oncogenesis, and altered tumor biology. The KRAS-variant has been found to be a strong biomarker of treatment response in many cancers, yet seems to act fundamentally differently from tumor-acquired KRAS mutations. Here, we show that this mutation predicts a positive response to anti-EGFR monoclonal antibody therapy in a large cohort of patients with metastatic colorectal cancer, without any benefit of additional chemotherapy for these patients. Cell line reporter studies also shed light on the functional biology of this mutation. These findings bring this powerful mutation one step closer to helping direct therapy for patients with metastatic colorectal cancer, allowing improved outcome and personalized treatment.

Although tumor-acquired KRAS mutational status testing is now mandatory for the initiation of anti-EGFR mAb treatment, approximately 50% to 65% of the patients with metastatic colorectal cancer with KRAS wild-type (WT) tumors still derive no benefit from these treatments, implying that additional genetic determinants of resistance, or perhaps sensitivity, exist (7, 14–16). Mounting evidence from retrospective studies suggest that the BRAF V600E mutation also confers resistant to anti-EGFR mAbs (14, 17–20), and, although not entirely clear yet, it also seems that PIK3CA-mutant tumors derive no little benefit from anti-EGFR mAb treatment (14, 21–23).

miRNAs, discovered almost 20 years ago (24), are an abundant class of highly conserved, endogenous, noncoding small RNA molecules, 18 to 25 nucleotides in length, which negatively regulate gene expression by binding to partially complementary sites in the 3’-untranslated region (UTR) of their target mRNAs (25, 26). The binding of miRNAs to their target mRNAs is critical for the regulation of mRNA levels and subsequent protein expression, and this regulation can be affected by SNPs or mutations in miRNA target sites in the 3’-UTR of genes. Such 3’-UTR variants appear to play an important role in human diseases like cancer (or conferring an increased risk for certain diseases, such as cancer; refs. 27, 28). A rapidly accelerating area of research is the systematic genomic evaluation of these sites, which are emerging as potential powerful biomarkers in the growing area of personalized medicine (29, 30). Such variants seem to affect not only gene expression but also tumor biology, drug response, and drug resistance (31–33), likely due to the critical role of miRNAs in managing the response to cytotoxic cancer therapy (33).

The lethal-7 (let-7) family of miRNAs was among the first discovered, and their differential expression has been found in a number of cancers (34). The KRAS oncogene is a validated direct target of the let-7 miRNA family, as let-7 induces KRAS downregulation upon binding to the 3’-UTR of the KRAS mRNA (34, 35). Recently, a functional variant was identified in a let-7 complementary site (LCS6) in the KRAS 3’-UTR mRNA (36). This variant (rs61764370) consists of a T to G base substitution and has been found to alter the binding capability of mature let-7 to the KRAS mRNA, resulting in both increased expression of the KRAS oncogenic protein and its downstream pathways (37), as well as altered let-7 miRNA levels in vivo, possibly due to a negative feedback loop. Consistent with the oncogenic nature of KRAS, the variant G allele has been shown to confer an increased risk of non–small cell lung cancer in moderate smokers (36), triple-negative breast cancer (37), and, in a subset of women, ovarian cancer (38, 39). More recently, significantly worse survival and platinum resistance was found in patients with ovarian cancer with the G allele (40). These findings indicate the functional and clinical significance of the KRAS 3’-UTR LCS6-variant.

In the metastatic colorectal cancer anti-EGFR mAb therapy setting to date, the KRAS LCS6-variant has been evaluated in four studies with selected populations and with contradicting (conflicting) results (30, 41–43). Graziano and colleagues (41) found within a KRAS and BRAF WT patients’ population treated with salvage irinotecan–cetuximab combination therapy that LCS6-variant carriers had a statistically significant worse progression-free survival (PFS) and overall survival (OS). In Sebio and colleagues (43), again patients with metastatic colorectal cancer treated primarily with irinotecan and cetuximab mAb were significantly more likely to be nonresponders (P = 0.004); however, in this study the KRAS LCS6-variant did not predict a different outcome in a cohort of people treated with non–mAb-based treatment, suggesting that the predictive properties of this mutation were primarily for cetuximab. In contrast to these studies, in a study where patients were given salvage cetuximab monotherapy (30), KRAS LCS6-variant carriers exhibited a longer PFS and OS, and had a better objective response rate (ORR). In addition, in the most recent study (42) of 180 patients with metastatic colorectal cancer receiving Nordic FLOX (bolus 5-fluorouracil/folinic acid and oxaliplatin) versus 355 patients receiving Nordic FLOX + cetuximab in the NORDIC-VII trial (NCT00145314), there were no significant differences in outcome for KRAS LCS6-variant patients. However, in fact, the addition of cetuximab seemed to improve response rate for LCS6-variant carriers more than nonvariant carriers (from 35% to 57% vs. 44% to 47%); however, the difference was not statistically significant [interaction P = 0.16]. These conflicting results have led investigators to question if different chemotherapy agents given in addition to cetuximab could impact the response to cetuximab for KRAS LCS6-variant patients (44).

Here, our goal was to clarify the role of this mutation as a biomarker of cetuximab response by evaluating the KRAS...
LCS6-variant along with other molecular markers (KRAS and BRAF) in a series of 512 patients with metastatic colorectal cancer who underwent either salvage anti-EGFR mAb monotherapy or mAbs in combination with chemotherapy. We show in our patient cohort as well as in cell lines that the KRAS LCS6-variant allele predicts a positive response to mAb monotherapy, without any additional benefit of cytotoxic chemotherapy.

Materials and Methods

Patients' characteristics

A total of 559 patients with metastatic colorectal cancer, 300 treated in the University Hospital of Leuven with anti-EGFR mAb monotherapy or mAb in combination with chemotherapy, as well as 148 patients from the Universite Paris Descartes treated with cetuximab-based salvage combination chemotherapy (14), and 111 previously published patients with metastatic colorectal cancer (30) treated with cetuximab monotherapy after failing fluoropyrimidine, irinotecan, and oxaliplatin containing regimens (30, 45), had tumor tissue available and amenable for analysis of the KRAS LCS6-variant. The mutational status of the KRAS and BRAF genes in the above mentioned patients’ populations has been previously published (14, 30). KRAS LCS6-variant status was successfully determined in 512 of the 559 patients with metastatic colorectal cancer tested. Molecular characteristics were correlated with ORR, PFS, and OS.

Genetic analyses

Formalin-fixed, paraffin-embedded normal tissue from the patients’ specimens was macroscopically dissected using a scalpel blade and DNA was isolated as previously described (14, 30). DNA was amplified using, as previously described (25), a custom-made Taqman genotyping assay (Applied Biosystems) designed specifically to identify the T or variant (rs61764370) with the forward primer: 50-CCGAGGTCTGTCGAA-30, reverse primer: 50-GCCAGGCTGGTCTCGAA-30, reporter probe: 50-CAAGTGATTCACCCAC- 30. The two-tailed Fisher exact test was used to compare proportions between nonvariant (46) TT patients and LCS-variant (TG and GG) patients. PFS and OS were estimated by the Kaplan–Meier method, and their association with genotypes was tested with the log-rank test. The association of genotypes with objective response was determined by contingency table and the Fisher exact test. To fully explore the possible influence of the KRAS LCS6-variant, analysis was performed in the whole metastatic colorectal cancer population, in the patients harboring no tumor-acquired mutations in the KRAS and BRAF genes (double WT population) and in the KRAS-mutated population. The level of significance was set at a two-sided value of 0.05. All statistical tests were performed using the statistical package SPSS version 13.

Luciferase reporter analysis

KRAS 3′-UTR reporter constructs were created by amplifying the entire KRAS 3′-UTR from cal27 cells (heterozygous for the LCS6-variant) using the following primers:

Forward: CGTATGACTCGAGATAACATTGTACCTTTTTC-TTAAGGCATACT
Reverse: ATGAAGCGCCGCGCTAGGAGTAGCATGACGTTCATG-ACAAAA.

The amplicons were subcloned into the XhoI and NotI sites in the 3′-UTR of the Renilla luciferase gene of the psiCHECK2 dual-luciferase vector (Promega). Reporters with the LCS6-variant (G allele) and without the variant (T allele) were confirmed by sequencing.

Dual-luciferase reporters (50 ng) containing either the KRAS 3′-UTR T allele or G allele were transfected into HCT-116 cells (grown at log phase), in a 24-well plate using Lipofectamine 2000 according to the manufacturer’s protocol. After a 16-hour incubation, the cells were lysed and lysates were analyzed for dual-luciferase activities by quantitative titration using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Renilla luciferase was normalized to firefly luciferase. The mean and SD of 4 independent experiments performed in duplicate were graphed. The P value was calculated by the Students t test.

For drug response analysis, dual-luciferase reporters (500 ng) were transfected into HCT-116 cells (grown at log phase) in a 6-mm plate using Lipofectamine 2000 according to the manufacturer’s protocol. Following a 6-hour transfection, the cells were trypsonized and replated at a density of 5.0 × 10⁴ cells per well in a 24-well plate along with the indicated amount of each drug. Following a 16-hour incubation, the cells were lysed and lysates were analyzed for dual-luciferase activities as described above. The mean and SD of independent experiments performed in duplicate were graphed.

Statistical analyses

The distribution of genotypes was in Hardy–Weinberg equilibrium, with a P value of 0.8. Because of the low frequency of homozygotes for the variant allele, patient samples that were either heterozygous (TG) or homozygous (GG) for the variant allele were considered positive for the KRAS LCS6-variant and entered the analyses as one group. PFS and OS were measured as previously described (14, 30).

The two-tailed Fisher exact test was used to compare proportions between nonvariant (46) TT patients and LCS-variant (TG and GG) patients. PFS and OS were estimated by the Kaplan–Meier method, and their association with genotypes was tested with the log-rank test. The association of genotypes with objective response was determined by contingency table and the Fisher exact test. To fully explore the possible influence of the KRAS LCS6-variant, analysis was performed in the whole metastatic colorectal cancer population, in the patients harboring no tumor-acquired mutations in the KRAS and BRAF genes (double WT population) and in the KRAS-mutated population. The level of significance was set at a two-sided P value of <0.05. All statistical tests were performed using the statistical package SPSS version 13.

Results

KRAS LCS6-variant in the entire patient cohort

In the 512 patients with metastatic colorectal cancer, 102 (19.9%) patients were heterozygous for the KRAS LCS6-variant and 7 (1.3%) patients homozygous for the variant, thus 109 (21.2%) had the KRAS LCS6-variant overall. KRAS tumor-acquired mutations in codons 12, 13, and 61 were found in 184 patients (33%) and the BRAF V600E mutation in 29 patients (5.3%). All patients received
anti-EGFR mAb-based salvage treatment. There were no statistically significant differences found between KRAS LCS6-variant and nonvariant carriers for sex or age at diagnosis. The characteristics of the 559 patients have been previously published (14, 30) and are also presented here in Supplementary Table S1.

As shown in Table 1, the distribution of the KRAS LCS6-variant genotypes was different among patients harboring tumor-acquired KRAS and BRAF mutations. Specifically, although the percentage of patients with the KRAS LCS6-variant was the same in KRAS WT and mutant groups (20% in each), variant patients were found twice as frequently in the BRAF V600E-mutant group versus in the BRAF-WT group (20%), resulting in a statistically significant difference ($P = 0.030$).

**Outcome and survival analysis in the entire patient cohort**

In the cohort as a whole, there were no significant differences in median PFS or OS between the nonvariant patients and the LCS6-variant patients (Supplementary Fig. S1A and S1B). Similarly, there were no differences in PFS or OS in the double (KRAS and BRAF) WT or in the KRAS-mutated patients’ cohorts comparing LCS6-variant and nonvariant patients. Finally, there were no significant correlations about response ($n = 483$) and skin rash ($n = 359$) with the LCS6-variant and nonvariant patients in the whole and in the double WT patients’ cohorts (Supplementary Table S3).

In the cohort as a whole however, in univariate analysis, tumor-acquired KRAS mutations [hazard ratio (HR), 1.688; $P = 0.0001$; 95% confidence interval, CI, 1.395–2.042], BRAF mutations (HR, 2.206; $P = 0.0001$; 95% CI, 1.501–3.243), and type of treatment (HR, 1.748; $P = 0.0001$; 95% CI, 1.450–2.108) were correlated with PFS and OS. Multivariate analysis revealed that the above factors have an independent association with decreased PFS and OS (Supplementary Table S4), and thus were incorporated into the below analysis.

**PFS with monotherapy versus combination treatment**

Next, we separately analyzed patients that received mAb monotherapy versus mAb combination therapy. From 501 patients with known treatment, 160 (32%) received anti-EGFR mAbs as monotherapy and 341 (68%) were treated with multiple chemotherapy combinations plus EGFR mAb therapy. Of the monotherapy patients, 32 (20%) had the LCS6-variant, and of the combination treatment patients, 75 (22%) had the LCS6-variant (NS). There were no significant differences in tumor-acquired KRAS and BRAF mutations between patients that received anti-EGFR mAb monotherapy versus combination therapy (Supplementary Table S2).

The median PFS of the whole monotherapy-treated patients’ population was 10.43 weeks (95% CI, 7.73–13.12 weeks). There was a statistically significant benefit of monotherapy for the LCS6-variant patients versus nonvariant patients, with a PFS of 16.86 weeks (95% CI, 10.2–23.51 weeks) versus 7.85 weeks (95% CI, 3.897–11.817 weeks; Fig. 1A; $P = 0.019$, log-rank test). The median PFS of the whole combination therapy patients’ population was 18 weeks (95% CI, 15.87–20.12 weeks), and there was no statistically significant difference observed between the LCS6-variant versus nonvariant patients [18 weeks (95% CI, 9.97–26.02 weeks) vs. 18.43 weeks (95% CI, 16.16–20.69 weeks); Fig. 1B; $P = 0.760$, log-rank test]. There was strong evidence for an interaction effect for PFS ($P = 0.051$).

Interestingly, there was no improved PFS for LCS6-variant patients that received mAb therapy [16.86 weeks (95% CI, 8.55–25.18 weeks)] versus combination therapy [18 weeks (95% CI, 13.37–22.64 weeks); Fig. 1C; $P = 0.291$, log-rank test]. In contrast, there was a significant benefit in PFS with the addition of chemotherapy for nonvariant patients ($P < 0.0001$, log-rank test), 7.86 weeks for monotherapy (95% CI, 3.9–11.82 weeks) versus 19.29 weeks for combination therapy (95% CI, 17–21.58 weeks; Fig. 1D). Of note, there was no significant difference in median PFS for monotherapy-treated LCS6-variant patients versus combination-treated nonvariant patients.

In the double (KRAS and BRAF) WT patients’ population, the median PFS of the monotherapy-treated patients was 12 weeks (95% CI, 8.38–15.61 weeks), and again a statistically significant difference was observed between nonvariant patients and LCS6-variant patients [10.43 weeks (95% CI, 6.74–14.11 weeks) vs. 18 weeks (95% CI, 5.16–30.83 weeks); Fig. 2A; $P = 0.039$, log-rank test]. The PFS for the combination therapy–treated patients was 28.71 weeks (95% CI, 24.98–32.45 weeks), and no statistically significant difference ($P = 0.39$, log-rank test) was observed between the nonvariant patients and LCS6-variant patients [28.3 weeks (95% CI, 24.15–32.45 weeks) vs.

**Table 1.** Distribution of the KRAS 3’-UTR LCS6 genotypes according to mutation, clinical, and demographic data in the cohort of patients with metastatic colorectal cancer

<table>
<thead>
<tr>
<th>TG or TT or GG</th>
<th>N (%)</th>
<th>TT or GG N (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median, min–max)</td>
<td>61 (22–89)</td>
<td>61 (37–80)</td>
<td>0.654</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>229 (57.4)</td>
<td>59 (54.6)</td>
<td>0.662</td>
</tr>
<tr>
<td>Female</td>
<td>170 (42.6)</td>
<td>49 (45.4)</td>
<td></td>
</tr>
<tr>
<td>KRAS status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>138 (36.3)</td>
<td>36 (34.6)</td>
<td>0.818</td>
</tr>
<tr>
<td>WT</td>
<td>242 (63.7)</td>
<td>68 (65.4)</td>
<td></td>
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<tr>
<td>BRAF status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>17 (4.3)</td>
<td>11 (10.2)</td>
<td>0.030</td>
</tr>
<tr>
<td>WT</td>
<td>379 (95.7)</td>
<td>97 (89.8)</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monotherapy</td>
<td>128 (32.1)</td>
<td>32 (29.6)</td>
<td>0.726</td>
</tr>
<tr>
<td>Combination</td>
<td>271 (67.9)</td>
<td>76 (70.4)</td>
<td></td>
</tr>
</tbody>
</table>

![Table 1.](attachment:image)

**Clinical Cancer Research**

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28.85 weeks (95% CI, 14.82–42.87 weeks); Fig. 2B]. Here, again, there was no significant improvement (P = 0.061, log-rank test) in PFS for LCS6-variant patients that received mAb monotherapy [18 weeks (95% CI, 5.1–30.8 weeks)] versus combination therapy [28.85 weeks (95% CI, 14.83–42.87 weeks); Fig. 2C], whereas there was improvement in PFS for nonvariant patients (P < 0.0001, log-rank test) that received mAb monotherapy [10.43 weeks (95% CI, 6.75–14.15 weeks)] versus combination therapy [28 weeks (95% CI, 24.1–31.8 weeks); Fig. 2D]. Again, there was no significant difference in median PFS between LCS6-variant patients receiving mAb monotherapy and nonvariant patients receiving combination therapy (18 vs. 28.8 weeks).

OS analysis correlated with treatment

The median OS of the whole monotherapy patients' population was 33.14 weeks (95% CI: 26.70–39.57 weeks), and no statistically significant difference (P = 0.139, log-rank test) was observed between the nonvariant patients and the LCS6-variant patients [28.85 weeks (95% CI, 22.53–35.18 weeks) vs. 45 weeks (95% CI, 35.02–54.97 weeks); Fig. 3A]. The median OS of the whole combination therapy patients' population was 44 weeks (95% CI, 40.06–47.93 weeks), and no statistically significant difference (P = 0.759, log-rank test) was observed between the nonvariant patients and the LCS6-variant patients [44 weeks (95% CI, 40.06–47.93 weeks) vs. 43 weeks (95% CI, 29.8–56.2 weeks); Fig. 3B]. For OS, the interaction term was clearly nonsignificant (P = 0.248).

There was no significant difference in OS for LCS6-variant patients that received mAb monotherapy [45 weeks (95% CI, 35–55 weeks)] versus combination therapy [43 weeks (95% CI, 29.8–56.2 weeks); Fig. 3C; P = 0.574, log-rank test], yet there was a significant benefit for OS with the addition of chemotherapy for nonvariant patients [mAb monotherapy 28.86 weeks (95% CI, 22.53–35.18 weeks) vs. combination therapy 44 weeks (95% CI, 40–47.93 weeks); P < 0.0001, log-rank test; Fig. 3D]. Again, the difference in OS for LCS6-variant patients receiving...
monotherapy and nonvariant patients receiving combination therapy was nonsignificant.

In the double (KRAS and BRAF) tumor WT patients' population, the median OS of the monotherapy patients was 37 weeks (95% CI, 30.82–43.17 weeks). A trend toward a statistically significant difference was observed between the nonvariant patients [35.71 weeks (95% CI, 32–39.4 weeks)] and the LCS6-variant patients [55.43 weeks (95% CI, 36.98–73.87 weeks; Fig. 4A; \( P = 0.087 \), log-rank test]. In this population, the median OS of the combination therapy patients was 55 weeks (95% CI, 48.3–61.7 weeks), and there was no statistically significant difference (\( P = 0.649 \), log-rank test) between the nonvariant patients [57 weeks (95% CI, 49.4–64.6 weeks)] and the LCS6-variant patients [54 weeks (95% CI, 45.46–62.53 weeks; Fig. 4B). Again, there was no significant improvement (\( P = 0.705 \), log-rank test) in OS for LCS6-variant patients that received mAb monotherapy [55.43 weeks (95% CI, 37–73.87 weeks)] versus combination therapy [54 weeks (95% CI, 45.47–62.54 weeks); Fig. 4C], whereas there was improvement in OS for nonvariant patients (\( P < 0.0001 \), log-rank test) that received mAb monotherapy [35.71 weeks (95% CI, 32–39.4 weeks)] versus combination therapy [57 weeks (95% CI, 49.4–64.6 weeks); Fig. 4D]. Again, the difference in OS for LCS6 G variant patients receiving monotherapy and KRAS WT patients receiving combination therapy was nonsignificant.

The LCS6-variant is not prognostic in KRAS- and BRAF-mutated patients

In the KRAS- and BRAF-mutated patients' population, no statistical significant differences in PFS or OS were observed in patients treated with either anti-EGFR mAb monotherapy or with mAbs in combination with chemotherapy (data not shown). Median PFS times were identical between LCS6-variant and nonvariant patients, with no significant improvement (\( P = 0.641 \), log-rank test) between PFS for LCS6-variant patients that received mAb monotherapy [6 weeks (95% CI, 0–13.25 weeks)] versus combination therapy [12 weeks (95% CI, 6.45–17.56 weeks); Supplementary Fig. S2A]. However, there was a significant improvement in PFS for nonvariant patients treated with combination therapy.
therapy. Likewise, for OS, there was no significant difference ($P = 0.303$, log-rank test) in OS for LCS6-variant patients that received mAb monotherapy [28.43 weeks (95% CI, 9.47–47.39 weeks)] versus combination therapy [23 weeks (95% CI, 10.8–35.19 weeks); Supplementary Fig. S2C], whereas there was a difference in OS for nonvariant patients ($P = 0.002$, log-rank test) that received mAb monotherapy [21.29 weeks (95% CI, 15–27.55 weeks) versus combination therapy [31 weeks (95% CI, 25.65–36.34 weeks); Supplementary Fig. S2D].

**The LCS6-variant and response to mAb therapy**

From the whole population of 483 patients that were evaluable for both response and LCS6-variant genotyping, 147 (30.4%) had received anti-EGFR mAb as monotherapy and 336 (69.6%) with multiple chemotherapy combinations. In the monotherapy group, 123 (83.6%) patients were nonresponders [stable disease and progressive disease (PD)], and 24 (16.4%) were responders [partial response (PR) and complete response (CR)]. There were significantly more LCS6-variant patients in the monotherapy responders group versus the nonresponders group (11/24 vs. 19/123; Fisher exact test, $P = 0.002$). In the combination with chemotherapy group, 252 (75%) patients were nonresponders (stable disease and PD) and 84 (25%) were responders (PR and CR). There was no statistically significant difference between the proportion of the nonvariant and LCS6-variant patients in these groups (Fisher exact test, $P = 0.1$).

In the 270 double (KRAS and BRAF) WT populations, 90 (33.3%) received anti-EGFR mAb as monotherapy and 180 (66.6%) with multiple chemotherapy combinations. In the
monotherapy group, 71 (78.8%) patients were nonresponders (stable disease and PD), and 19 (21.2%) were responders (PR and CR). Again, there were significantly more LCS6-variant patients in the responders versus the nonresponders groups (9/19 vs. 11/71; Fisher exact test, $P = 0.010$). In the combination with chemotherapy group, 102 (56.6%) patients were nonresponders (stable disease and PD) and 78 (43.4%) were responders (PR and CR). There was no statistically significant difference between the proportion of nonvariant and LCS6-variant patients in the groups (Fisher exact test, $P = 1$).

The effect of the LCS6-variant on gene expression upon therapy exposure

Because patients with the LCS6-variant in this analysis were sensitive to EGFR mAb monotherapy, it suggested that they are not EGFR-independent, as tumor-acquired KRAS mutant patients are (30). To better understand how these patients may respond differently to mAb monotherapy, we created dual-luciferase reporters containing the entire KRAS $^{3^l}$-UTR with the LCS6-variant (G allele) or without the variant (T allele). We used this system to test the hypothesis that mAb therapy and chemotherapy may differentially impact expression of KRAS for those with the LCS6-variant allele versus the nonvariant allele.

We found, as has been previously reported in other cell lines (36), that the LCS6-variant allele (G allele reporter) displayed 1.8-fold higher expression at baseline in HCT-116 colon cancer cells when compared with the nonvariant allele (T allele reporter, Fig. 5A). This finding supports previous evidence that this is a functional mutation that permits KRAS overexpression in tumors. Next, we exposed these cells to cetuximab, and found that although there was

![Figure 4. LCS6-variant patients do not have improved OS with the addition of chemotherapy for double WT patients. A, median OS according to the KRAS $^{3^l}$-UTR LCS6 SNP genotype status in the double (KRAS and BRAF) WT patients' population treated with anti-EGFR mAb monotherapy as salvage treatment. B, median OS according to the KRAS $^{3^l}$-UTR LCS6 SNP genotype status in the double (KRAS and BRAF) WT patients' population treated with anti-EGFR mAb-based combination chemotherapy as salvage treatment. C, median OS according to type of therapy in the double (KRAS and BRAF) WT KRAS $^{3^l}$-UTR LCS6 SNP carriers. D, median OS according to type of therapy in the double (KRAS and BRAF) WT non-KRAS $^{3^l}$-UTR LCS6 SNP carriers.](image-url)
experiments performed in duplicate. The results are expressed as the relative expression of the LCS-variant versus the nonvariant allele. Graphed is the mean and SD of the mean for two nonvariant allele.

Figure 5B. The LCS6-variant allele leads to exposure to irinotecan (Fig. 5B). These results indicate that the LCS6-variant allele compared to the nonvariant allele with exposure to 5-fluorouracil. In contrast, we saw little to no change in expression of the LCS6-variant allele with exposure to 5-fluorouracil. The finding was supported by cell line studies indicating that the LCS6-variant allele mediates the selection of less differentiated and more aggressive clones that harbor BRAF mutations, and perhaps our cohort was more advanced. In addition, there could be a selective pressure to develop KRAS or BRAF mutations in the presence of the LCS6-variant, depending on exposure to specific therapies, and prior therapy likely differed between our two studies.

Discussion
Here, we have shown a statistically significant improvement in median PFS for all LCS6-variant patients with metastatic colorectal cancer treated with anti-EGFR mAb monotherapy. This improved prognosis is not enhanced by the addition of chemotherapy, and in fact, LCS6-variant patients seemed to experience no benefit from the addition of chemotherapy to anti-EGFR mAb therapy. This finding is in contrast to nonvariant patients, who derived a significant benefit from the addition of chemotherapy to anti-EGFR mAbs across all cohorts, and only then achieved comparable outcomes as those of LCS6-variant patients. This clinical finding was supported by cell line studies indicating that the LCS6-variant allele responds differently than the nonvariant allele in response to chemotherapy and anti-EGFR mAb exposure, with increased expression and likely further dependence on the KRAS pathway (47). A different distribution of the LCS6 genotypes according to the KRAS and BRAF mutational status was observed in our population of patients with metastatic colorectal cancer than that observed in prior reports. LCS6-variant patients were equally likely to have acquired KRAS tumor mutations as not, but, LCS6-variant patients were significantly more likely to be in the BRAF-mutated group. In a previously studied metastatic colorectal cancer population similar to ours, Graziano and colleagues (41) found an increased prevalence of the LCS6-variant in the KRAS mutant, but not in BRAF-mutant patients (41). Although one explanation for our different results could be that we used tumor DNA for the majority of testing, this seems unlikely, since, it has previously been well documented that the genotype of normal and tumor tissue is the same in LCS6-variant patients (36). Another hypothesis could be that in the later stages of colorectal cancer carcinogenesis, the LCS6-variant allele mediates the selection of less differentiated and more aggressive clones that harbor BRAF mutations, and perhaps our cohort was more advanced. In addition, there could be a selective pressure to develop KRAS or BRAF mutations in the presence of the LCS6-variant, depending on exposure to specific therapies, and prior therapy likely differed between our two studies.

The finding that this single base pair change in the 3'-UTR of KRAS leads to a significant difference in both baseline expression as well as response to chemotherapy in a luciferase reporter construct is intriguing. Although tumor-acquired KRAS mutations are always turned on, these cell line reporter studies further indicate how fundamentally different this mutation is than a simple tumor-acquired KRAS mutation. By their nature, miRNA-binding disrupting mutations, such as the KRAS LCS6-variant, are dependent on trans-activating factors, such as miRNAs, that change in response to stress. It has been known for several years that miRNAs are used to dynamically regulate the response to cytotoxic cancer therapy (33). It is perhaps not surprising...
therefore that a mutation such as the LCS6-variant would be predictive of cancer treatment response, as cancer treatments will lead to changes in the very factors that regulate the mutation, and subsequent downstream gene and pathway expression. However, further molecular studies of the exact mechanisms by which this mutation alters response to EGFR mAb treatment are still required in tissue and animal models.

Recently, two large studies of patients with colon cancer investigating outcome found that the LCS6-variant allele predicted a good prognosis, especially when in combination with tumor-acquired mutations in KRAS, in both early-(48) and late-stage (49) patients. These authors hypothesized that at least in early-stage colon cancer, the LCS6-variant plus KRAS mutations could lead to too much KRAS and tumor cell senescence. On the basis of our cell line data, indicating that anti-EGFR mAb monotherapy leads to significantly higher KRAS expression, as does 5FU, but not irinotecan, we hypothesize that this may be a viable explanation of the very favorable anti-EGFR mAb monotherapy response in advanced KRAS LCS6-variant patients as well. It does further support the hypothesis that the combination of therapy delivered with anti-EGFR mAb monotherapy is critical, as there seems to be no benefit of additional chemotherapy in our study, and in fact chemotherapy could possibly be a detriment to patients with LCS6-variant metastatic colorectal cancer.

As is also true for other cancers, an important step in the development of colorectal cancers seems to be the deregulation of miRNAs. Over the past few years, miRNAs have been brought to the central stage of molecular oncology and have substantially changed the way we view and understand gene regulation (50). The KRAS LCS6-variant was the first mutation in a miRNA-binding site to be implicated in cancer risk, and although it certainly will not be the last (36), it seems to also play a significant predictive role that could guide therapy decisions. Our findings here suggest that patients carrying the LCS6-variant are biologically different than nonvariant patients, have a higher probability of benefit from anti-EGFR mAb monotherapy, and deserve prospective clinical studies to determine what, if anything, they should receive in addition to cetuximab treatment in the metastatic colorectal cancer setting.

Disclosure of Potential Conflicts of Interest

J.B. Weidhaas has ownership interest (including patents) in and is a consultant/advisory board member for Mirati Dk. H.-J. Lenz is a consultant/advisory board member for Bristol-Myers Squibb and Merck. P. Laurent-Puig is a consultant/advisory board member for Amgen and Merck Serono. O. Bouche reports receiving speakers bureau honoraria from Amgen and is a consultant/advisory board member for Merck Serono. S. Tejpar is a consultant/advisory board member for Merck Serono. No potential conflicts of interest were disclosed by the other authors.

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References


The KRAS-Variant Predicts Cetuximab Response in Colon Cancer


A *let-7* microRNA-Binding Site Polymorphism in *KRAS* Predicts Improved Outcome in Patients with Metastatic Colorectal Cancer Treated with Salvage Cetuximab/Panitumumab Monotherapy


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