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Clinical Cancer Research

Validation of miR-31-3p Expression to Predict Cetuximab Efficacy When Used as First-Line Treatment in *RAS* Wild-Type Metastatic Colorectal Cancer

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Abstract

Purpose: MiR-31-3p expression has been shown to be associated with response to anti-EGFR therapy. We investigated the predictive role of this biomarker in the FIRE-3 study population, including its ability to differentiate outcomes between patients receiving anti-EGFR and anti-VEGF therapy.

Experimental Design: MiR-31-3p expression was measured in primary tumors obtained from 340 patients with *RAS* WT mCRC enrolled in the FIRE-3 Trial. This included 164 patients randomized to receive FOLFIRI plus cetuximab (FOLFIRI+Cetux) and 176 to FOLFIRI plus bevacizumab (FOLFIRI+Beva). Patients were divided into subgroups defined by low or high miR-31-3p expression using a prespecified cut-off and by treatment arm. Analyses were performed to assess treatment efficacy by subgroup. Overall survival (OS) and progression-free survival (PFS) were analyzed using Kaplan–Meier curves and Cox regression models. Investigator-assessed objective response (iOR), early tumor shrinkage at 6 weeks (ETS), and centrally reviewed objective response (cOR) were analyzed using logistic regression models. The predictive value of miR-31-3p expression level was assessed through a treatment interaction test using multivariate models adjusted for potential confounding factors.

Results: Low miR-31-3p expressers benefited from cetuximab compared with bevacizumab for PFS [HR, 0.74; 95% confidence interval (CI), 0.55–1.00; $P = 0.05$], OS (HR, 0.61; 95% CI, 0.41–0.88; $P < 0.01$), iOR (OR, 4.0; 95% CI, 1.9–8.2; $P < 0.01$), ETS (OR, 4.0; 95% CI, 2.1–7.7; $P < 0.01$ and cOR (OR, 4.9; 95% CI, 2.3–10.5; $P < 0.01$) in multivariate analyses. There was no difference in outcomes for high expressers between treatment arms. MiR-31-3p expression level was predictive of treatment effect for PFS ($P = 0.03$), OS ($P = 0.05$), iOR ($P = 0.02$), ETS ($P = 0.04$), and cOR ($P < 0.01$).

Conclusions: MiR-31-3p expression level was validated as a predictive biomarker of cetuximab therapy efficacy for patients with *RAS* WT mCRC. *Clin Cancer Res; 1–8. ©2018 AACR.*

Introduction

The use of the anti-EGFR antibodies cetuximab and panitumumab in the treatment of metastatic colorectal cancer (mCRC) is restricted to patients with extended *RAS* wild-type (WT) tumors because no clinical benefit is derived from either therapy in patients with *RAS* mutant tumors (1). Unfortunately, patients with *RAS* WT treated with anti-EGFR therapy only experience an up to 70% objective response rate, correlating to approximately 30% of patients not responding to treatment (2, 3). Because the side-effect profile for anti-EGFR therapy, especially skin toxicity, is significant, further personalization of treatment is needed to maximize benefit-to-risk ratio for these drugs.

MicroRNAs are small noncoding RNA molecules that are key regulators of gene expression and have been shown to play a critical role in many cancers (4). MicroRNA expression levels can serve as biomarkers for diagnostic, prognostic or predictive purposes in CRC (5, 6). Mir-31 is frequently upregulated in CRC tumors compared with normal corresponding tissue (7). Upregulation of the mature forms of mir-31, miR-31-3p, and miR-31-5p is associated with advanced disease and poor response to anti-EGFR therapy based on several small retrospective studies (8–12).

The current study was planned using the REporting recommendations for tumor MARKer prognostic studies (REMARK; ref. 13) with the aim to validate the predictive role of miR-31-3p expression level for the efficacy of the anti-EGFR antibody cetuximab in first-line treatment of mCRC.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

A Post-Hoc Analysis of the FIRE-3 (AIO KRK-0306) Trial

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Translational Relevance

The current standard of care for first-line treatment options in patients with metastatic colorectal cancer (mCRC) includes the combination of chemotherapy with an anti-EGFR or anti-VEGF monoclonal antibody. The presence of a *RAS* mutation in the primary tumor precludes the use of anti-EGFR antibodies in this patient population. By measuring miR-31-3p expression in tumors in the RAS wild-type population of the FIRE-3 trial, the present study was designed to examine if this biomarker enables the identification of patients more likely to benefit from cetuximab compared with bevacizumab in regard to survival outcomes and objective response to therapy in mCRC. Our analysis demonstrated that the expression level of miR-31-3p is predictive of cetuximab efficacy for patient survival and response-related outcomes as compared with bevacizumab when both are combined with chemotherapy for the treatment of mCRC.

Patients and Methods

Study design and participants

The FIRE-3 (AIO KRK-0306; NCT00433927) prospective, randomized trial investigated the efficacy of first-line treatment with FOLFIRI plus either cetuximab or bevacizumab in 592 patients with *KRAS* Exon-2 WT mCRC (14). Although progression-free survival (PFS) was comparable between treatment arms, a statistically significant improvement in overall survival (OS) was demonstrated for the cetuximab arm compared with the bevacizumab arm. Extended *RAS* analysis performed retrospectively on patients treated in the FIRE-3 study revealed 400 subjects bearing a *RAS* WT tumor (2). Tumor material was available for 343 subjects with miR-31-3p expression measurable for 340 patients (miR-population or "miR-pop"; Fig. 1). The retrospective analysis of tumor material was approved by the ethics committee of the Ludwig-Maximilians-University Munich (IRB No. 186-15).

MiR-31-3p expression analyses

A pathologist (D. Neureiter) reviewed all samples and the area of tumor was marked for subsequent macrodissection. For each sample, 5 formalin-fixed, paraffin-embedded (FFPE) slides of 5-μm thickness were scratched in the tumor area and total RNAs were extracted using the FFPE miRNeasy Extraction Kit (Qiagen) according to the manufacturer's instructions. Specific quantification of microRNA miR-31-3p expression was performed on retrotranscribed RNA using specific TaqMan predesigned assays on ABI7900HT Real-Time PCR System as described by Ramon and colleagues (15). A previously reported cutoff value (1.36) for miR-31-3p expression level was used to define patients as being either low or high expressers (15).

Statistical methodology

The primary objective of the predefined statistical analysis plan was to demonstrate the superiority of FOLFIRI+Cetux versus FOLFIRI+Beva regarding OS and PFS in the low-expressers subgroup, defined as patients with miR-31-3p expression level below the prespecified cutoff value. If the primary objective was achieved, miR-31-3p expression predictivity of treatment efficacy would then be tested. Secondary endpoints were: (i) investigator-assessed objective response (iOR), (ii) centrally-reviewed early tumor shrinkage (ETS) at 6 weeks, and (iii) centrally reviewed objective response (cOR), as binary variables. The cut-off to define response for iOR, ETS, and cOR was tumor shrinkage equal or higher than 30% from baseline, evaluated according to RECIST 1.0 (16). Patients with missing data were excluded from secondary endpoints analyses.

OS and PFS time to first event were analyzed using Kaplan–Meier curves. Treatment effect on survival outcomes was estimated with hazard ratios with their confidence intervals computed through Cox regression model. iOR, ETS, and cOR treatment odds ratios were calculated using logistic regression models.

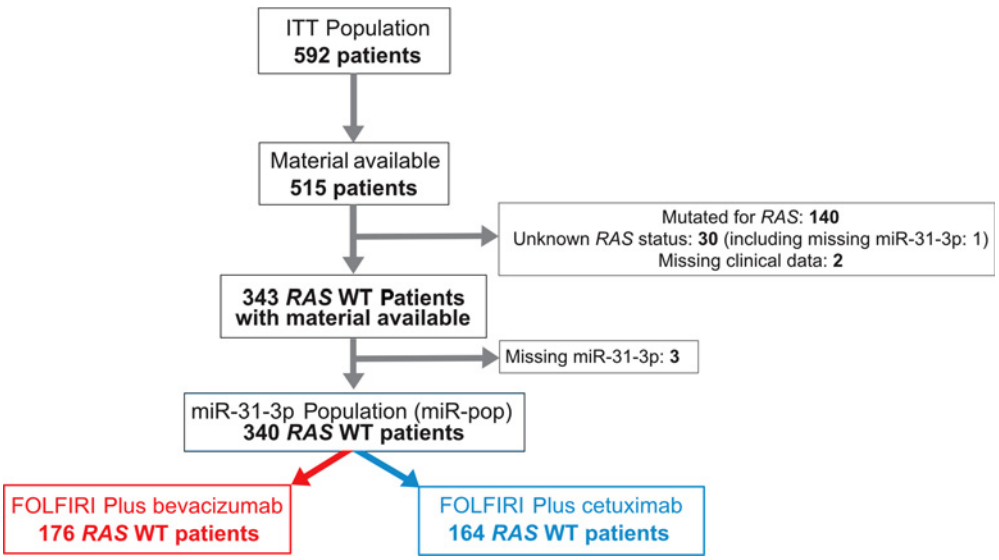

Figure 1.
FIRE-3 patients disposition for miR-31-3p analyses.

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Each baseline characteristic was tested in a univariate analysis, using biologic treatment as the only covariate, to assess its prognostic value on survival outcomes. A multivariate model was then developed using all variables found to be significant in univariate analyses (threshold of $P = 0.01$ in miR-pop and $P = 0.05$ in subpopulations), removing variables which were not significant ($P \geq 0.05$) in the multivariate model. MiR-31-3p expression as a quantitative variable (log of expression level) was tested in univariate analyses only. Mir-31-3p expression level as a binary variable (low vs. high expression) was tested alone and with adjustment for selected confounding factors.

The predictivity of miR-31-3p expression level on treatment efficacy was tested by adding an interaction term with treatment in the final multivariate prognostic model. Prognostic effect was considered significant at the threshold of $P < 5\%$ and predictive effect at the threshold of $P < 10\%$ as specified in the Statistical Analysis Plan. The other significant prognostic variables were also tested for predictivity.

Analyses were performed using R version 3.3.2 and SAS version 9.4.

Results

Out of 515 subjects with available biological samples, 343 subjects were WT for *KRAS* and *NRAS* exons 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codons 117 and 146) (ref. 2). Among the 340 subjects composing the miR-pop, 176 were treated with FOLFIRI+Beva and 164 with FOLFIRI+Cetux (Fig. 1).

Distributions of baseline characteristics in the miR-pop were comparable by treatment arms and homogeneous with distributions observed in the FIRE-3 final *RAS* WT population (2) (Table 1). One-third of the miR-pop were high expressers (Supplementary Fig. S1). Although high expressers more often had right-sided and BRAF V600E mutated tumors compared with low expressers, both subgroups were comparable for other characteristics (Table 2). In low and high miR-31-3p expresser subgroups, treatment arms were balanced for all baseline characteristics (Supplementary Table S1).

Although OS was significantly longer in subjects treated with FOLFIRI+Cetux [HR, 0.71; 95% confidence interval (CI), 0.53–0.93; $P = 0.01$] in miR-pop (Supplementary Fig. S2), there was no difference in PFS between the 2 treatment arms (HR, 0.92; 95% CI, 0.73–1.16; $P = 0.47$; Supplementary Fig. S3). This finding is consistent with results obtained on FIRE-3 final *RAS* WT population (PFS: HR, 0.97; 95% CI, 0.78–1.20; $P = 0.77$ and OS: HR, 0.70; 95% CI, 0.54–0.90; $P = 0.006$; ref. 2).

Prognostic value of miR-31-3p expression on OS and PFS

MiR-31-3p expression level (quantitative and binary), BRAF mutational status, ECOG score, tumor sidedness, and number of metastatic sites were prognostic for PFS and OS (Supplementary Figs. S4 and S5, respectively) in univariate analyses after adjustment for treatment effect.

MiR-31-3p low expressers had a longer PFS and OS compared with high expressers (median PFS in months: 11.1; 95% CI, 10.1–12.2 vs. 7.8; 95% CI, 6.9–9.3; HR, 1.43; 95% CI, 1.11–1.83; $P < 0.01$; median OS in months: 30.3; 95% CI, 26.1–36.4 vs. 20.3; 95% CI, 14.8–23.8; HR, 1.76; 95% CI, 1.32–2.34;

Table 1. Baseline characteristics in FOLFIRI bevacizumab and FOLFIRI cetuximab, in FIRE-3 *RAS* WT and miR-pop populations

	FIRE-3 *RAS* WT		miR-pop	
	Bevacizumab (*n* = 201)	**Cetuximab (*n* = 199)**	**Bevacizumab (*n* = 176)**	**Cetuximab (*n* = 164)**
Gender				
Female	68 (34%)	53 (27%)	61 (35%)	42 (26%)
Male	133 (66%)	146 (73%)	115 (65%)	121 (74%)
Age, y				
≤65	105 (52%)	104 (52%)	90 (51%)	88 (54%)
>65	96 (48%)	95 (48%)	86 (49%)	76 (46%)
Median (y)	65	64	65	64
BRAF status				
WT	176 (88%)	176 (88%)	155 (88%)	143 (87%)
Mutant	25 (12%)	23 (12%)	21 (12%)	21 (13%)
Number of metastatic sites				
1	82 (41%)	85 (43%)	77 (44%)	73 (45%)
≥2	118 (59%)	112 (57%)	99 (56%)	90 (55%)
Median	2	2	2	2
ECOG score				
0	109 (54%)	107 (54%)	93 (53%)	87 (53%)
1	89 (44%)	89 (45%)	80 (45%)	76 (46%)
2	3 (2%)	3 (1%)	3 (2%)	1 (1%)
Tumor sidedness				
Left+rectum	149 (75%)	157 (81%)	132 (79%)	128 (80%)
Right	50 (25%)	38 (19%)	36 (21%)	31 (20%)
miR-31-3p				
Low (<cutoff)	—	—	121 (69%)	108 (66%)
High (≥cutoff)	—	—	55 (31%)	56 (34%)
Mean (SD)	—	—	1.52 (2.43)	1.58 (2.28)
Median	—	—	0.58	0.73
Min - Max	—	—	0.01–20	0.01–17.31

NOTE: Percentages calculated on patients without missing data (13 missing for sidedness, 1 missing for gender, 1 for number of metastatic sites). Percentages calculated on patients without missing data (miR-pop: 13 missing for sidedness, 1 missing for gender, 1 for number of metastatic sites/FIRE-3 *RAS* WT: 2 missing for number of metastatic sites, 6 missing for sidedness).

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Table 2. Baseline characteristics by miR-31-3p expression subgroups

	Low ($n = 229$)	High ($n = 111$)
Gender		
Female	65 (29%)	38 (34%)
Male	163 (71%)	73 (66%)
Age, y		
≤65	121 (53%)	57 (51%)
>65	108 (47%)	54 (49%)
Median (y)	64	64
BRAF status[a]		
WT	218 (95%)	80 (72%)
Mutant	11 (5%)	31 (28%)
Number of metastatic sites		
1	103 (45%)	47 (42%)
≥2	125 (55%)	64 (58%)
Median	2	2
ECOG score		
0	123 (54%)	57 (51%)
1	104 (45%)	52 (47%)
2	2 (1%)	2 (2%)
Tumor sidedness[a]		
Left + rectum	191 (88%)	69 (63%)
Right	26 (12%)	41 (37%)

NOTE: Percentages calculated on patients without missing data (13 missing for sidedness, 1 missing for gender, 1 for number of metastatic sites).
[a]$P < 0.05$.

$P < 0.01$). MiR-31-3p expression as a quantitative variable was also prognostic of OS and PFS ($P < 0.01$).

In the multivariate model that included BRAF mutational status, sidedness, number of metastatic sites and ECOG score as additional variables to miR-31-3p and treatment arm, the prognostic value of miR-31-3p was still significant for OS (HR, 1.43; 95% CI, 1.05–1.96; $P = 0.02$) but not for PFS (HR, 1.11; 95% CI, 0.85–1.45; $P = 0.43$).

Differential treatment benefit in low versus high miR-31-3p subgroups and miR-31-3p expression level predictivity of treatment effect on OS and PFS

In the low-expressers subpopulation (Fig. 2), a benefit of FOLFIRI+Cetux was observed for PFS (HR, 0.74; 95% CI, 0.55–1.00; $P = 0.05$) and OS (HR, 0.61; 95% CI, 0.41;0.88; $P < 0.01$) in the multivariate model. Median PFS was increased by 1.3 months and median OS by 12 months in patients treated with FOLFIRI+Cetux compared with patients treated with FOLFIRI+Beva. Except for female patients for PFS (HR, 1.07, NS), and patients with right-sided tumor for OS (HR, 1.11; NS), all subgroups benefitted from FOLFIRI+Cetux compared with FOLFIRI+Beva (HR < 1) for PFS and OS, and the only significant treatment interaction was the number of metastatic sites for PFS ($P = 0.08$; Supplementary Figs. S6 and S7 for PFS and OS, respectively). Conversely, in the high-expressers subgroup (Fig. 2), no statistically significant benefit of FOLFIRI+Cetux was observed for OS (HR, 1.12; 95% CI, 0.69–1.79; $P = 0.65$) and median PFS was increased by 1.7 months in patients treated with FOLFIRI+Beva compared with patients treated with FOLFIRI+Cetux. This benefit of FOLFIRI+Beva for PFS in high expressors was not statistically significant (HR, 1.32; 95% CI, 0.87–2.02; $P = 0.19$). Significant ($P < 0.10$) treatment interactions were observed for *BRAF* status and number of metastatic sites for OS, and tumor sidedness for both PFS and OS (Supplementary Figs. S8 and S9 for PFS and OS, respectively).

In multivariate analyses, a significant interaction was found in miR-pop between miR-31-3p expression level and treatment arm for both PFS (interaction test $P = 0.03$) and OS (interaction test $P = 0.05$) demonstrating miR-31-3p expression level was predictive of treatment effect for PFS and OS. With the exception of tumor sidedness ($P < 0.01$) and BRAF status ($P = 0.08$) for OS, no other predictive factors were found in these analyses (Supplementary Fig. S4 for PFS and Supplementary Fig. S5 for OS).

Analysis of secondary endpoints

The predictive value of miR-31-3p expression level on treatment response was investigated for iOR, ETS at 6 weeks and cOR (Table 3). MiR-31-3p expression level was predictive of treatment effect for all 3 endpoints (interaction tests: $P = 0.02$ for iOR, $P = 0.04$ for ETS and $P < 0.01$ for cOR). In low expressers, iOR was 86.7% in the FOLFIRI+Cetux arm compared with 62.5% for patients in the FOLFIRI+Beva arm (OR, 4.0; 95% CI, 1.9–8.2; $P < 0.01$). In high expressers, there was no significant difference for iOR between either the FOLFIRI+Beva arm and the FOLFIRI+Cetux arm (iOR, 55.8% and 63.5% respectively, OR, 1.3; 95% CI, 0.6–2.9; $P = 0.57$). Similar patterns were observed for cOR and ETS at 6 weeks, with an improved treatment observed in low expressers treated with FOLFIRI+Cetux compared with FOLFIRI+Beva (OR, 4.9; 95% CI, 2.3;10.5; $P < 0.01$ for cOR and OR, 4.0; 95% CI, 2.1;7.7; $P < 0.01$ for ETS at 6 weeks), and no difference in high expressers (OR, 0.8; 95% CI, 0.3–1.9; $P = 0.56$ for cOR, and OR, 1.3; 95% CI, 0.5–3.2; $P = 0.60$ for ETS at 6 weeks). In patients treated with FOLFIRI+Cetux, a linear correlation was found between miR-31-3p expression and tumor shrinkage considered as continuous variables (correlation coefficient = 0.28, $P = 0.002$), whereas no correlation was observed in the FOLFIRI+Beva arm (Supplementary Fig. S10). The interaction between treatment arm and miR-31-3p expression level for tumor shrinkage was statistically significant ($P = 0.03$).

Analysis of the interaction between miR-31-3p expression and tumor sidedness

Because tumor sidedness has been recently proposed as a predictive factor of anti-EGFR antibody efficacy in mCRC, we performed analyses of PFS, OS and iOR by miR-31-3p expression level and tumor sidedness (Supplementary Tables S2 and S3). MiR-31-3p expression was found to be higher in right-sided tumors than in tumors located in the left colon or rectum ($P < 0.05$; Table 2). In right-sided tumor subjects, miR-31-3p expression level was predictive of PFS and OS (interaction miR-31-3p with treatment: $P = 0.04$ for PFS and $P = 0.06$ for OS). In left-sided tumors, no predictive effect of miR-31-3p on PFS and OS could be detected. Regarding iOR, the benefit of adding cetuximab to FOLFIRI as compared with bevacizumab was restricted to low expressers, regardless of the tumor sidedness. Response rates in low-expresser patients treated with FOLFIRI+Cetux were 89.3% for left-sided and 72.7% for right-sided tumors, compared with 63.0% and 53.9%, respectively, for patients treated with FOLFIRI+Beva. In high expressers, response rates were similar for patients treated with FOLFIRI+Cetux and FOLFIRI+Beva in both left-sided (67.6% vs. 61.3%) and right-sided tumors (53.3% vs. 50.0%).

Discussion

Precision medicine requires robust biomarkers that identify patients with a high probability of benefiting from a specific therapy. This is important for first-line decisions in mCRC because

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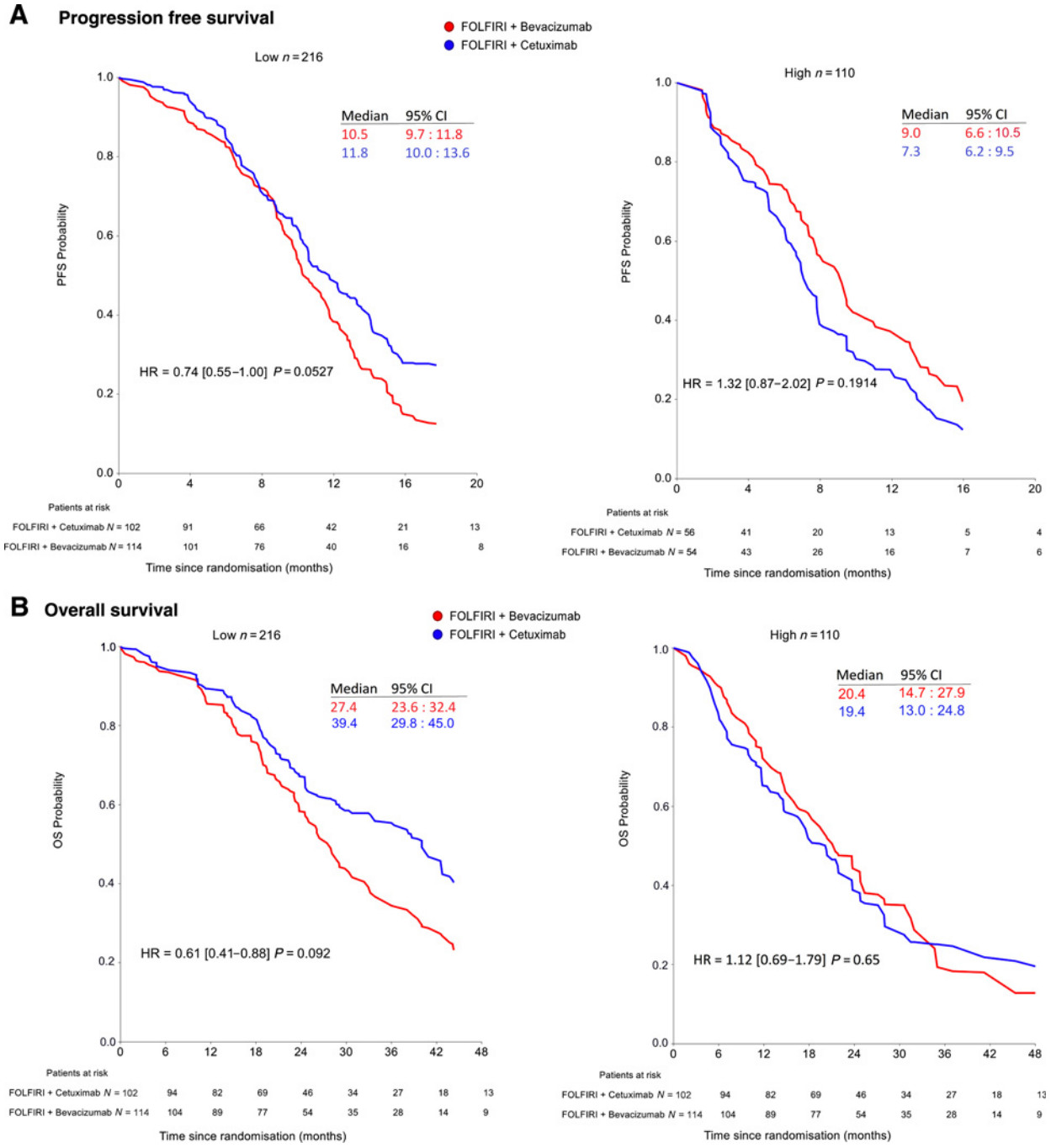

Figure 2.

Treatment effect on PFS (**A**) and OS (**B**) separately by "low" and "high" expressers. Survival curves and median times and hazard ratios estimated through multivariate Cox regression (95% confidence intervals, *P* values) adjusted on BRAF mutational status, tumor sidedness, ECOG score and number of metastatic sites for PFS and low expressers; BRAF mutational status and tumor sidedness for PFS and high expressers; tumor sidedness, ECOG score and number of metastatic sites for OS and low expressers; BRAF mutational status, tumor sidedness and number of metastatic sites for OS and high expressers.

20% to 45% of patients do not receive second-line treatment due to early death or deterioration (17, 18). The FIRE-3 phase-III study is an optimal trial for the validation of a biomarker for first-line anti-EGFR therapy in patients with RAS WT mCRC since the anti-EGFR arm was tested against an anti-VEGF arm in a prospective, randomized fashion with both arms using the same chemotherapeutic backbones.

Consistent with previous reports (10–12), we demonstrated that miR-31-3p expression level within the *RAS* WT population is predictive of anti-EGFR therapy efficacy relative to tumor

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Table 3. Treatment effect on iOR, cOR and ETS: rates by treatment arms and odds ratios estimated through bivariate and multivariate logistic regressions with treatment and miR-31-3p expression level as covariates

Endpoint	miR-pop expression group (*n*)[a]	Univariate OR [95% CI] *P* value	Multivariate OR [95% CI] *P* value (adjusted for)	miR-pop group by treatment interaction test *P* value (adjusted for)	Response rates (total number of patients) FOLFIRI + bevacizumab	Response rates (total number of patients) FOLFIRI + cetuximab
iOR	High ($n = 104$)	1.4 [0.6–3.0] $P = 0.42$	1.3 [0.6–2.9] $P = 0.57$ (1)	$P = 0.02$ (4)	55.8% (52)	63.5% (52)
	Low ($n = 202$)	3.9 [1.9–8.0] $P < 0.01$	4.0 [1.9–8.2] $P < 0.01$ (2)		62.5% (112)	86.7% (90)
cOR	High ($n = 98$)	0.9 [0.4–2.0] $P = 0.81$	0.8 [0.3–1.9] $P = 0.56$ (1)	$P < 0.01$ (1)	55.3% (47)	52.9% (51)
	Low ($n = 181$)	5.0 [2.4–10.4] $P < 0.01$	4.9 [2.3–10.5] $P < 0.01$ (2)		54.8% (104)	85.7% (77)
ETS (6 weeks)	High ($n = 98$)	1.4 [0.6–3.4] $P = 0.42$	1.3 [0.5–3.2] $P = 0.60$ (1)	$P = 0.04$ (4)	27.7% (47)	35.3% (51)
	Low ($n = 181$)	3.8 [2.0–7.1] $P < 0.01$	4.0 [2.1–7.7] $P < 0.01$ (3)		25.0% (104)	55.8% (77)

NOTE: Multivariate analysis adjusted for (i) BRAF mutational status and ECOG score (ii) ECOG score (iii) number of metastatic sites (iv) BRAF mutational status number of metastatic sites and ECOG score in the multivariate model. Patients with missing response were excluded.
[a]Patients with missing response were excluded.

response, PFS and OS, after adjustment for confounding factors such as *BRAF* mutational status and tumor sidedness. Patients with *RAS* WT tumors that had low miR-31-3p expression levels experienced a significant benefit from FOLFIRI+Cetux with a significantly longer PFS and OS compared with patients receiving FOLFIRI+Beva. On the contrary, there was no significant benefit of FOLFIRI+Cetux versus FOLFIRI+Beva in patients with high miR-31-3p expression levels. Although the 1.3 month PFS difference in favor of FOLFIRI+Cetux versus FOLFIRI+Beva in low miR-31-3p expressors was significant, the 1.7-month difference in PFS in the opposite direction favoring FOLFIRI+Beva in high expressors was not statistically significant due to the lesser number of patients in the high-expressor subgroup compared with the low-expressor subgroup. The above results suggest a potential benefit of using anti-EGFR therapy as first-line treatment in patients whose tumors have low miR-31-3p expression level. The results of our study also validate the pre-specified cutoff value used to define low and high miR-31-3p expressers.

We also found that miR-31-3p expression level was associated with early treatment efficacy such as ETS and OR. Patients with low miR-31-3p expression level had an ETS rate of 56% and a cOR of 86%. These findings open the possibility of identifying patients with borderline resectable metastases who may have a higher likelihood of benefiting from surgery since higher tumor response has been shown to be correlated with higher secondary resection rates (19).

Primary tumor sidedness has recently been discussed as a marker for clinical decision making in metastatic colorectal cancer since patients with right colon tumors have a worse prognosis than those with tumors in the left colon (20). Our analyses revealed that patients with low miR-31-3p expression and left-sided tumors benefited the most from FOLFIRI+Cetux compared with FOLFIRI+Beva. Although high miR-31-3p expressors with right-sided tumors benefited more from FOLFIRI+Beva in regard to OS and PFS, low miR-31-3p expression level was predictive of an improved response rate for FOLFIRI+Cetux compared with FOLFIRI+Beva, regardless of sidedness. This suggests that if response is the primary goal of first-line treatment, miR-31-3p levels may provide a tool for identifying patients with right-sided tumors who would respond more favorably to FOLFIRI+Cetux versus FOLFIRI+Beva. Conversely, patients with left-sided tumors benefited from FOLFIRI+Cetux vs. FOLFIRI+Beva regardless of their level of miR-31-3p expression, although the response rate was higher in low expressors. Because the analysis of tumor sidedness as a part of the present study was done on a post-hoc basis, additional studies are needed to better define the impact of tumor sidedness on the predictive effect of miR-31-3p, especially in patients with left-sided tumors.

Mir-31 has been shown to promote CRC progression and mir-31 plays a significant role in activating the RAS signaling pathway through the inhibition of RAS p21 GTPase activating protein 1 (RASA1) translation, thereby improving colorectal cancer cell growth and stimulating tumorigenesis (8, 9). Moreover, different studies have shown that high miR-31 expression is associated with KRAS and BRAF mutational status in pancreatic cancer cells (21). MiR-31 host gene transcription and mature miR-31 expression have been shown to be induced by the RAS oncogenic pathway (22, 23). In our study, we indeed confirmed that miR-31-3p expression level was higher in BRAF mutant (mean ± SD = 2.51 ± 3.02 vs. 0.53 ± 3.95, Student *t* test $P < 0.01$) and in RAS mutant (data not shown) populations compared with the RAS/BRAF WT population (24). Furthermore, it has been shown that EGFR suppresses the maturation process of pre-mir-31 via the phosphorylation of AGO2, in response to hypoxic stress (Supplementary Fig. S11). Low miR-31-3p expression could be a consequence of the regulation of pre-mir-31 maturation by an EGFR activated pathway, driving tumor sensitivity to anti-EGFR therapy. Contrary to this, high miR-31-3p expression could be the witness of the tumor's EGFR independency and subsequently to its resistance to anti-EGFR therapy.

Following REMARK criteria (13), the predictive value of a pre-specified cut-off for miR-31-3p expression level was validated during the present study. On the basis of these results, we propose performing miR-31-3p testing in conjunction with *RAS* mutational analyses would add information useful to clinicians for aiding in the identification of the optimal treatment selection and maximizing the benefit-to-risk ratio for the use of first line anti-EGFR in their patients with mCRC.

There are several potential limitations for our study. The use of FOLFIRI as the only backbone chemotherapy in FIRE-3 trial

and the potential for an interaction between cetuximab and irinotecan impacting the predictive value of miR-31-3p regarding anti-EGFR agent efficacy cannot be excluded. Also, although baseline characteristics were well balanced between treatment arms and analyses were conducted according to a prespecified plan, a potential bias due to the retrospective nature of the study could exist. The extension of these analyses to other chemotherapy backbones and/or other anti-EGFR molecules remains to be studied. Demonstrating similar findings following the analysis of tumor samples from other randomized controlled trials, or from real-life cohorts would enable the possibility to generalize our conclusions to all patients with mCRC treated in first line.

In conclusion, using a prespecified cutoff, miR-31-3p expression level was validated as a predictive biomarker of the efficacy of first-line cetuximab therapy when use in the treatment of mCRC patients. Patients with low miR-31-3p–expressing tumors had a significantly greater benefit from FOLFIRI plus cetuximab when compared with patients treated FOLFIRI plus bevacizumab. This suggests that miR-31-3p expression level is a useful biomarker to further personalize the treatment of mCRC. Additional studies are warranted to determine whether similar findings would be observed in patients with mCRC treated in first line with FOLFOX plus EGFR-antibody therapy.

Disclosure of Potential Conflicts of Interest

P. Laurent-Puig holds ownership interest (including patents) in Integragen, and is a consultant/advisory board member for Amgen, Boerhinger Ingelheim, Merck Serono, Roche, and Sanofi. V. Heinemann reports honoraria from Amgen, Baxalta, Merck, Roche, Sanofi, Servier, and SIRTEX, consulting and advisory roles for Amgen, Baxalta, Boehringer, Lilly, Merck, Roche, Sanofi, Servier, and SIRTEX, travel and accommodation expenses from Baxalta, Merck, Roche, SIRTEX, and research funding for his institution from Amgen, Merck, Pfizer, Roche, Sanofi, and SIRTEX. A. Jung reports receiving speakers bureau honoraria from Amgen, Merck Serono, Novartis, and Roche, and is a consultant/advisory board member for Amgen, Biocartis, Merck Serono, Novartis, and Roche. S. Stintzing reports personal fees from Amgen, Bayer AG, Lilly, Merck Serono, Roche AG, Sanofi, and Takeda. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Neureiter, R. Thiébaut, S. Stintzing

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Laurent-Puig, M.-L. Grisoni, F. Liebaert, D. Neureiter, F. Montestruc, Y. Gaston-Mathe, S. Stintzing

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