Hsa-miR-31-3p Expression Is Linked to Progression-free Survival in Patients with KRAS Wild-type Metastatic Colorectal Cancer Treated with Anti-EGFR Therapy

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Abstract

Purpose: To identify microRNAs (miRNA) that predict response to anti-EGFR antibodies in patients with wild-type KRAS metastatic colorectal cancer (mCRC).

Experimental Design: miRNA profiling was performed in a training set of 87 patients with mCRC refractory to chemotherapy treated with anti-EGFR antibodies. This included 33 fresh-frozen (FF) and 35 formalin-fixed paraffin-embedded (FFPE) samples retrospectively collected and 19 prospectively collected samples. An independent validation cohort consisting of 19 FF and 26 FFPE prospectively collected samples from patients with mCRC treated with anti-EGFR antibodies was used to confirm our findings.

Results: After screening the expression of 1,145 miRNAs in FF samples from the training set, we identified that hsa-miR-31-3p expression level was significantly associated with progression-free survival (PFS). Statistical models based on miRNA expression discriminated between high and low risk of progression for both FF and FFPE samples. These models were confirmed in the validation cohort for both FF [HR, 4.1; 95% confidence interval (CI), 1.1–15.3; *P* = 0.04] and FFPE samples (HR, 2.44; 95% CI, 1.1–5.4; *P* = 0.028). The percentage of variation of RECIST criteria in the validation series was significantly associated with the expression level of hsa-miR-31-3p (*r*² = 0.49; *P* = 0.0035) and risk status determined by hsa-miR-31-3p expression level (*P* = 0.02; Kruskal–Wallis rank test). Nomograms were built and validated to predict PFS depending on hsa-miR-31-3p expression level. Following in vitro studies, we identified 47 genes regulated by hsa-miR-31-3p.

Conclusion: Hsa-miR-31-3p seems to be a new mCRC biomarker whose expression level allows for the identification of patients with wild-type KRAS mCRC who are more likely to respond to anti-EGFR therapy.

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Introduction

With more than 1.2 million new cases in 2008, colorectal cancer is the third most common form of cancer worldwide (1, 2). Nearly 20% of patients are diagnosed at an advanced stage with metastatic disease (1) and over half will ultimately develop metachronous metastases. The recent development of targeted therapies has improved outcomes in patients with advanced colorectal cancer. Cetuximab and panitumumab, two monoclonal antibodies, which neutralize the extracellular domain of EGFR, have demonstrated effectiveness in terms of both response and survival when used as first, second or third-line chemotherapy. Although anti-EGFR agents are routinely used in clinical practice, their use is limited to 60% of patients who have KRAS wild-type tumors (2) because randomized-controlled studies have shown activating mutations of this oncogene were predictive of resistance to these agents (3–12).

Because response to anti-EGFR therapy is seen in less than 40% of patients with KRAS wild-type tumors (13), additional factors are needed to help patient selection for this therapy to avoid the prescription of ineffective, expensive, and potentially harmful therapy. In the present study, we identify and validate a microRNA (miRNA), hsa-miR-31-3p, as a marker for predicting survival of patients treated with anti-EGFR monoclonal antibodies with a KRAS wild-type mCRC. Furthermore, we provide information on the genes regulated by this miRNA.

Translational Relevance

Anti-EGFR therapy has demonstrated effectiveness in patients with metastatic colorectal cancer (mCRC). The use of these agents is limited to a population of patients for whom the tumors show KRAS wild-type status because randomized-controlled studies have demonstrated that only this subgroup of patients can benefit from this therapy. Only 40% of patients with KRAS wild-type tumors treated with anti-EGFR agents respond to this treatment; therefore, additional markers are needed to help patient selection to avoid the prescription of ineffective, expensive, and potentially harmful therapy. In the present study, we identify and validate a microRNA (miRNA), hsa-miR-31-3p, as a marker for predicting survival of patients treated with anti-EGFR monoclonal antibodies with a KRAS wild-type mCRC. Furthermore, we provide information on the genes regulated by this miRNA.

Patients and Methods

Patients

Four cohorts of patients with mCRC were included in this study (n = 132). Three were used as training sets and one as a validation set. All samples were obtained from studies that had previously received appropriate ethical committee approvals. Samples for the first training series (TS-Frozen1) were obtained from a retrospective collection of 33 patients, some of whom were reported on in a previously published series (13, 20). All patients were refractory to a FOLFOX or FOLFIRI regimen, refractory to these regimens plus anti-EGFR antibodies, or received panitumumab as a single agent.

Samples for the second training series (TS-Frozen2) were obtained from a prospective collection of patients treated by anti-EGFR antibodies alone or in combination with an irinotecan-based chemotherapy regimen. All of these patients were considered refractory to FOLFIRI regimen.

The third training set (TS-Frozen2) was made up of samples from a retrospective series of pooled Belgian and Finnish patients with mCRC. Some of these patients were a part of previously published study (32). All patients in this training set were considered refractory to a FOLFIRI regimen. Clinical interest because of the inability to set an appropriate threshold limit (28, 29).

MicroRNAs (miRNA) are small 19 to 25 nucleotides, noncoding RNAs that negatively regulate 30% of gene expression posttranscriptionally by inhibiting translation and degrading mRNAs. miRNAs control biologic processes such as cell proliferation, differentiation, angiogenesis, and apoptosis (30), while also acting as oncomiR or suppressor miRs depending of their target mRNA. miRNAs deregulation, through expression modification and point mutations, is involved in the occurrence of many types of cancer, including colorectal cancer (31). These abnormalities are of major interest and could serve for cancer diagnosis, prognosis, and chemotherapy response prediction.

This study aimed to develop a miRNA expression–based model for predicting survival of patients with KRAS wild-type metastatic colorectal cancer (mCRC) treated with anti-EGFR monoclonal antibodies.
demonstration of progression to oxaliplatin- and irinotecan-based chemotherapy regimens (33). This validation cohort was divided in two (Fig. 1A and B). The first cohort (VS-Frozen) consisted of patients with FF samples \((n=19)\) with the second cohort (VS-FFPE) consisting of patients with FFPE samples without FF samples available \((n=26)\) or with FF samples \((n=39)\).

**DNA and RNA extraction**

DNA and RNA extraction and mutation analysis was only performed on specimens with greater than 50% tumor component. DNAs were extracted from frozen matched tumor and nontumor tissue samples using the QIAamp DNA Mini Kit (Qiagen). Total RNAs were extracted from frozen tumors, cells or FFPE using the mirVanamiRNA Isolation Kit (Ambion), miRNeasy, and miRNeasy FFPE Extraction Kit (Qiagen), respectively (see Supplementary Methods).

**KRAS and BRAF mutation**

Methods of KRAS and BRAF mutation detection are described in the Supplementary Methods (13, 34).

**Screening of the miRNAs**

Global miRNA profiling was performed by labeling and hybridizing 750 ng of extracted RNA from 43 tumor frozen samples randomly chosen from TS-Frozen1 and TS-Frozen2 on Illumina Human v2 microRNA Expression Beadchips. Beadchips were scanned with the Illumina I-Scan Reader and data were imported into GenomeStudio data analysis software (Illumina), quantile-normalized and log₂-transformed. For expression analysis on TaqMan (Applied
provided for each patient with a log expression profile given of the Kaplan–Meier survival curves. A prognostic index is validation set series and whole series (training bootstrap resamples, then independent validation. The training set of patients. Internal validation relied on 150 the probability of progression-free survival (PFS) based on a Cox proportional regression hazard models, which predict generating an HR and a performance index (area under the analyses used a Cox proportional regression hazard model and principal component analysis performed on TS-Frozen1 using the Taq-
variate association between each miRNA and PFS allowed us to select a set of 11 miRNAs, for which Cox score statistics were found significant (P < 0.01; Supplementary Table S2 and Supplementary Fig. S1).

All miRNAs were tested for in the first and second training sets using TaqMan probes. Only one miRNA, hsa-miR-31-3p, showed a significant association with PFS on TS-Frozen1 and TS-Frozen2 with HRs being 1.8; 95% confidence interval (CI), 1.1–2.9; P = 0.01 and 2.5; 95% CI, 1.3–4.5; P = 0.002, respectively. In addition, a Super-PC analysis combining Cox proportional hazard model and principal component analysis performed on TS-Frozen1 using the Taq-Man expression level of miR-31–3p allowed us to define a prognostic score, computed by the formula: 0.178x – 1.36, where x is the TaqMan log-transformed expression of hsa-miR-31-3p, and a cutoff value of −0.031 dividing patients into two groups with a high and low risk of progression (Supplementary Fig. S2). The HR was 2.6; 95% CI, 1.15–5.8; P = 0.021; the PFS of high- and low-risk patients was 13 versus 31.4 weeks, respectively. This prognostic score was calculated and the cutoff value was applied on TS-Frozen2. The PFS of high- and low-risk patients was 9 versus 35.3 weeks, respectively (HR, 4.1; 95% CI, 1.3–13.2; P = 0.018; Fig 2A). The model was further validated for patients with FF sample in the PIMABI phase II study (validation set frozen, n = 19). The PFS of high- and low-risk patients was 23 versus 48.8 weeks, respectively (HR, 4.1; 95% CI, 1.1–15.3; P = 0.02; Fig 2B).

To be independent of a dichotomized variable, we built a nomogram based on Cox proportional hazards regression modeling patients PFS probability with the log-transformed hsa-miR-31-3p expression on the TS-Frozen1 (Fig. 3). The BRAF status, a known prognostic marker in mCRC, was added into the model. The nomogram was then tested on TS-Frozen2 and VS-Frozen. The nomogram demonstrated the ability to predict PFS with an AUROC superior to 0.70, whatever the time threshold tested (Supplementary Fig. S3).
In anticipation of the use of this marker in clinical practice, we evaluated its performance on RNA extracted from FFPE tissues. To validate the prognostic value of the expression of hsa-miR-31-3p on FFPE samples, we used two sets of patients in which FFPE tissue samples were available (TS-FFPE, n = 35 and VS-FFPE, n = 26). Patients from TS-FFPE were divided into two groups according to expression of hsa-miR-31-3p, as was done for frozen samples using a Cox model associated with a principal component analysis of hsa-miR-31–3p and a new cutoff value (C00.058). An HR for PFS of 3.2; 95% CI, 1.4–7.5; \( P = 0.008 \).

Finally, when we pooled the different series \( n = 132 \) taking into account the classification based on the expression of hsa-miR-31-3p (Supplementary Fig. S4). The OR of nonresponse in the high-risk patients was 4.9; 95% CI, 2–12.5; \( P < 0.001 \). We performed a multivariate analysis, including the ECOG performance status and the BRAF status in RAS wild-type patients \( n = 104 \). The PFS HR and OS HR between high- and low-risk patients were 2.3; 95% CI, 1.4–3.8; \( P = 0.008 \).

### Table 1. Baseline patient characteristics

<table>
<thead>
<tr>
<th>Patient data</th>
<th>Training set 1 ( (N = 33) )</th>
<th>Training set 2 ( (N = 19) )</th>
<th>Training set 3 ( (N = 35) )</th>
<th>Validation set 1a ( (N = 19) )</th>
<th>Validation set 1b ( (N = 26) )</th>
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<td>Gender</td>
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<tr>
<td>Male</td>
<td>24 (72.7%)</td>
<td>11 (57.9%)</td>
<td>16 (45.7%)</td>
<td>12 (63.2%)</td>
<td>18 (69.2%)</td>
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<td>Female</td>
<td>9 (27.3%)</td>
<td>8 (42.1%)</td>
<td>19 (54.3%)</td>
<td>7 (21.2%)</td>
<td>8 (30.8%)</td>
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<tr>
<td>Age</td>
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<td></td>
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<tr>
<td>Mean</td>
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<td>65.0</td>
<td>63.3</td>
<td>61.8</td>
<td>60.2</td>
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<td>(47.3–77.9)</td>
<td>(37–82)</td>
<td>(33.9–84.5)</td>
<td>(34–82)</td>
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<td>Chemotherapy regimen</td>
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<td></td>
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<td></td>
<td></td>
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<td>Cetuximab</td>
<td>0</td>
<td>0</td>
<td>2 (5.7%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cetuximab + irinotecan</td>
<td>24 (72.7%)</td>
<td>11 (57.9%)</td>
<td>16 (45.7%)</td>
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<td>0</td>
</tr>
<tr>
<td>Cetuximab + Xeloda</td>
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<tr>
<td>Cetuximab + FOLFIRI</td>
<td>5 (15.2%)</td>
<td>4 (21.1%)</td>
<td>4 (11.4%)</td>
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<td>0</td>
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<tr>
<td>Cetuximab + FOLFOX</td>
<td>2 (6.0%)</td>
<td>0</td>
<td>1 (2.9%)</td>
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<td>0</td>
</tr>
<tr>
<td>Panitumumab</td>
<td>0</td>
<td>4 (21.1%)</td>
<td>8 (22.9%)</td>
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<tr>
<td>Panitumumab + XELIRI</td>
<td>0</td>
<td>0</td>
<td>1 (2.9%)</td>
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<tr>
<td>Panitumumab + irinotecan</td>
<td>1 (3.0%)</td>
<td>0</td>
<td>3 (8.6%)</td>
<td>19 (100%)</td>
<td>26 (100%)</td>
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<td>Number of treatment lines before anti-EGFR therapy</td>
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<td>3</td>
<td>2</td>
<td>2</td>
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<td>(1–6)</td>
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<td>0</td>
<td>1 (5.3%)</td>
<td>2 (7.7%)</td>
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<tr>
<td>Response according to RECIST criteria</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial response</td>
<td>10 (30.3%)</td>
<td>4 (21.1%)</td>
<td>13 (37.1%)</td>
<td>7 (36.8%)</td>
<td>8 (30.8%)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>11 (33.3%)</td>
<td>8 (47.4%)</td>
<td>10 (28.6%)</td>
<td>6 (31.6%)</td>
<td>8 (30.8%)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>10 (30.3%)</td>
<td>6 (31.6%)</td>
<td>12 (34.3%)</td>
<td>5 (26.3%)</td>
<td>7 (26.9%)</td>
</tr>
</tbody>
</table>

In anticipation of the use of this marker in clinical practice, we evaluated its performance on RNA extracted from FFPE tissues. To validate the prognostic value of the expression of hsa-miR-31-3p on FFPE samples, we used two sets of patients in which FFPE tissue samples were available (TS-FFPE, \( n = 35 \) and VS-FFPE, \( n = 26 \)). Patients from TS-FFPE were divided into two groups according to expression of hsa-miR-31-3p, as was done for frozen samples using a Cox model associated with a principal component analysis to calculate a new prognostic score \( (0.093x - 0.407, where x is the FFPE TaqMan log-transformed expression of hsa-miR-31-3p) \) and a new cutoff value \( (0.058) \). An HR for PFS of 3.2; 95% CI, 1.4–7.5; \( P = 0.008 \).

Finally, when we pooled the different series \( n = 132 \) taking into account the classification based on the expression of hsa-miR-31-3p (Supplementary Fig. S4). The OR of nonresponse in the high-risk patients was 4.9; 95% CI, 2–12.5; \( P < 0.001 \). We performed a multivariate analysis, including the ECOG performance status and the BRAF status in RAS wild-type patients \( n = 104 \). The PFS HR and OS HR between high- and low-risk patients were 2.3; 95% CI, 1.4–3.8; \( P < 0.001 \) and 1.9; 95% CI, 1.2–3.1; \( P = 0.008 \).
significantly downregulated (fold change < 0.7; P < 0.05),
and 27 genes significantly upregulated by hsa-miR-31-3p
(fold change < 1.3; P < 0.05; Supplementary Table S3).

As the role of a miRNA includes degradation of its
transcript target, we studied whether our database predicted
the 47 downregulated genes as a hsa-miR-31-3p putative
target. Twenty-five of the genes were predicted to be putative
direct targets of hsa-miR-31-3p and displayed a good rank in
the prediction database. This number and the ranking of
genes were significant (P < 0.0001 for both by the permu-
tation test). Twenty-six of the upregulated genes were not
predicted to be direct targets of hsa-miR-31-3p. Although
one of the upregulated genes was predicted to be a direct
target, it was the last ranked target in the prediction
database.

The 25 putative direct and 27 indirect target genes were
validated on qRT-PCR. Out of these 52 genes, 45 displayed
an expression level comparable with the level obtained in
the array. When the expression of these genes was analyzed
in 39 tumor samples, we found a correlation between
hsa-miR-31-3p expression pattern and PFS for DBNDD2
(P = 0.02) and EPB41L4B (P = 0.009). Interestingly, both
genes displayed a negative correlation with hsa-miR-31-3p
expression levels: DBNDD2 (−0.5; P = 0.001) and
EPB41L4B (−0.3; P = 0.04; Supplementary Fig. S5).

Discussion

By using an assumption-free approach to identify miR-
NAs associated with PFS in patients with KRAS wild-type
mCRC treated by anti-EGFR therapy, we established for the
first time a link between hsa-miR-31-3p expression and the
risk of progression. All patients in our cohorts were refrac-
tory to the chemotherapy regimen used with anti-EGFR
therapy. We were able to define a robust threshold by
dividing patients in two groups according to their risk of
progression after anti-EGFR therapy. Our results indepen-
dently confirm those of a Finnish group (32) who demon-
strated a significant differential expression of hsa-miR-31-3p
between patients with stable and progressive disease,
even though we used a part of their patient set in TS-FFPE to
determine the threshold of the hsa-miR-31-3p in FFPE tissues.

The availability of multiple drugs for mCRC (e.g., panitumumab, cetuximab, and bevacizumab) underlines the need for better decision-making tools. The survival improvement of patients with mCRC often results from the use of these different drugs via a personalized approach or precise manner. The nomogram we developed, which predicts PFS on an individualized basis, provides such a decision-making tool. The accuracy of this nomogram on frozen samples was assessed by the calculation of the AUC that gives a value superior to 0.70, which can be considered as a good performance for a predictor. There is also a good correlation between hsa-miR-31-3p expression levels measured in frozen samples and those measured in FFPE samples, confirming the interest to use miRNAs as surrogate for wider gene expression signatures. Nevertheless, we needed to readjust the threshold of the hsa-miR-31-3p expression to divide the high- and low-risk progression groups for FFPE samples. Furthermore, although PFS can be considered a good surrogate marker of efficacy in well-designed randomized clinical trials, its value per se in retrospective series is unclear. We also investigated the impact of hsa-miR-31-3p with other critical variables such as response rate and OS. There was a significant link between response status (nonresponder vs. responder) and the classification in high- and low-risk patients according hsa-miR-31-3p expression as shown in the waterfall plot of the patients included in the PIMABI phase II study. We also validated this association in the whole series, the risk of nonresponse is five times more frequent in the high-risk patient group than in the low-risk patient group. Finally, we also showed a significant link between the OS and the hsa-miR-31-3p expression level in the whole series adjusted on the other prognostic variables (ECOG and BRAF mutational status). The high-risk patient group has a significant shorter survival than the patients belonging to the low-risk group.

Several miRNAs have been shown to be associated with response to chemotherapy in different types of cancers (37–39). To our knowledge, only one group has reported an association between the expression of a miRNA (hsa-miR-143) with PFS and cancer-specific survival in patients with mCRC treated by anti-EGFR alone or in combination with chemotherapy in refractory setting (40). The authors, however, failed to identify a link between the expression of this miRNA and response to anti-EGFR therapy, suggesting that this biomarker is more a prognostic marker than predictive, even if some relation with 5FU sensitivity have been described (41).

The specific role of the hsa-miR-31-3p has been investigated through in silico and in vitro approaches and the list of genes is not easy to interpret. The 25 genes putatively downregulated by the hsa-miR-31-3p do not give a clear picture to a regulatory mechanism of response to anti-EGFR therapy. A recent link between some specific regulation of miRNAs maturation and EGFR kinase activity has been emphasized and could enlighten our results (42). In hypoxic conditions the maturation of pre-miRNAs with long loop structures are dependent on the phosphorylation of AGO2 protein by EGFR. The phosphorylated AGO2 protein reduced its interaction with DICER protein leading to decrease significantly the loading of pre-miRNA or the expression of mature miRNA. Among the miRNAs regulated by this mechanism, the premature mir-31 is one of the most

Figure 4. Kaplan-Meier PFS curves and the waterfall plot for RECIST criteria for the whole series of validation (PIMABI phase II; n = 45). Patients are classified in high and low risk of progression according to the value obtained for FFPE samples for patients with FFPE samples (n = 39) and for FF samples for those with only FF samples (n = 6). The RECIST criteria were available only for 40 patients.
likely candidates. The absence of EGFR induced increased expression of miR-31 and inhibition of EGFR by tyrosine kinase inhibitors such as gefitinib. The high level of hsa-miR-31-3p expression in our patient’s tumors could be the result of an absence of EGFR response to hypoxia (43) and thus reduced benefit from anti-EGFR therapy. Among the genes derepressed by the inhibition of maturation of miRNA by EGFR response to hypoxia (42), two genes were found in common with our list of downregulated genes by the hsa-miR-31-3p: AMFR and EPB41L4B, the latter being also associated with PFS.

We were unable to demonstrate the predictive role of hsa-miR-31-3p expression in our study because there was no control arm consisting of patients without anti-EGFR therapy. In an attempt to assess the predictive nature of hsa-miR-31-3p, we first tested its prognostic value in the PETACC3 cohort (43, 45) and did not find a correlation between hsa-miR-31-3p expression and survival after relapse (HR, 0.93; P = 0.48; data not shown). These data were based on 124 KRAS and NRAS wild-type patients. Furthermore, in a recent study by Shen and colleagues (42), the expression of phosphorylated AGO2 protein has been shown to be associated with worse prognosis in patients with breast cancer, suggesting that maturation inhibition of miRNAs controlled by EGFR is a marker of aggressiveness in at least breast cancer patients. Our results are exactly at the opposite, suggesting that high expression of hsa-miR-31-3p is rather a predictive than a prognostic marker.

 Altogether, our results suggest that the expression hsa-miR-31-3p could be of help in the decision to add anti-EGFR therapy to common chemotherapy regimens in the population of KRAS wild-type patients by giving a probability of PFS, which will be of interest with the development of several targeted therapy in mCRC.

Disclosure of Potential Conflicts of Interest

R. Thiebaut, F. Liebaert, F. Rousseau, and B. Génin are employees of IntegraGen. J.-B. Bachet is a consultant/advisory board member for Amgen and Merck Serono. O. Bouché reports receiving speakers bureau honoraria from Amgen and is a consultant/advisory board member for Merck Serono. F. Bibeau reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Merck Serono. O. Bouché reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Merck Serono. P. Osterlund reports receiving a commercial research grant from Roche, speakers bureau honoraria from Prime Oncology, and is a consultant/advisory board member for Amgen, Bayer, Merck Serono, Roche, and Sanofi. T. André reports receiving speakers bureau honoraria from Amgen and is a consultant/advisory board member for Amgen and Merck Serono. P. Laurent-Puig is a consultant/advisory board member for Amgen, Merck Serono, and IntegraGen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Development of methodology: S. Imbeaud, R. Thiebaut, F. Rousseau, B. Génin, D. Le Corre, A. Didelot, S. Knuutila, P. Laurent-Puig

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-B. Thiebaut, K. Fontaine, F. Rousseau, J.-B. Bachet, B. Chibaudel, O. Bouché, B. Landi, F. Bibeau, K. Leroy, F. Penault-Llorca, J.-L. Van Laethem, S. Tejpár, N. Mosakhani, P. Osterlund, R. Ristamäki, V. Sarhadi, S. Knuutila, V. Boige, T. André, P. Laurent-Puig


Writing, review, and/or revision of the manuscript: G. Manceau, R. Thiebaut, F. Liebaert, B. Génin, M. Vincent, J.-B. Bachet, B. Chibaudel, O. Bouché, B. Landi, J.-L. Van Laethem, P. Demetter, S. Tejpár, S. Rossi, P. Osterlund, S. Knuutila, V. Boige, T. André, P. Laurent-Puig

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Thiebaut, J.-B. Bachet, B. Landi, P. Demetter, P. Laurent-Puig

Study supervision: G. Manceau, R. Thiebaut, F. Liebaert, T. André, P. Laurent-Puig

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