Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

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

Gilles Manceau[1,4], Sandrine Imbeaud[2], Raphaële Thiébaut[3], François Liébaert[3], Karine Fontaine[3], Francis Rousseau[3], Bérengère Génin[3], Delphine Le Corre[1], Audrey Didelot[1], Marc Vincent[1], Jean-Baptiste Bachet[4], Benoist Chibaudel[5], Olivier Bouché[10], Bruno Landi[6], Frédéric Bibeau[11], Karen Leroy[7], Frédérique Penault-Llorca[12], Jean-Luc Van Laethem[13], Pieter Demetter[14], Sabine Tejpar[15], Simona Rossi[16], Neda Mosakhani[17], Pia Österlund[18], Raija Ristamäki[20], Virinder Sarhadi[19], Sakari Knuutila[17,19], Valérie Boige[1,8], Thierry André[5], and Pierre Laurent-Puig[1,9]

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 FF 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. *Clin Cancer Res; 20(12); 3338–47. ©2014 AACR.*

Authors' Affiliations: [1]Université Paris Sorbonne Cité; INSERM UMR-S775 Bases Moléculaires de la réponse aux xénobiotiques; [2]INSERM UMR-S674 Genomique Fonctionnelle des Tumeurs; [3]Integragen S.A., Evry; [4]Université Pierre et Marie Curie; Assistance Publique-Hôpitaux de Paris, Hôpital Pitié Salpétrière; [5]Université Pierre et Marie Curie; Assistance Publique-Hôpitaux de Paris, Hôpital Saint-Antoine; [6]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, service d'Hépato-Gastro-Entérologie et d'Oncologie Digestive; [7]Université Paris-Est Créteil; Assistance Publique-Hôpitaux de Paris, Hôpital Henri Mondor, Créteil; [8]Department of Medicine, Institut Gustave Roussy, Villejuif; and [9]Department of Biology, Assistance Publique-Hôpitaux de Paris, European Georges Pompidou, Paris; [10]Université de Reims Champagne-Ardenne; Centre Hospitalier Universitaire de Reims, Reims; [11]Service d'Anatomo-Pathologie, Centre Val d'Aurelle Paul-Lamarque, Montpellier; [12]Université Clermont-Ferrand, Centre Jean Perrin, Clermont-Ferrand, France; [13]Department of Gastroenterology, GI Cancer Unit; [14]Department of Pathological Anatomy, Erasme University Hospital, Brussels; [15]Digestive Oncology Unit, University Hospital Gasthuisberg, Leuven, Belgium; [16]Bioinformatics Core Facility, Swiss Institute of Bioinformatics, Lausanne, Switzerland; [17]Haartman Institute, University of Helsinki; [18]Department of Oncology, Helsinki University Central Hospital and Helsinki University; [19]HUSLAB, Department of Pathology and Genetic Laboratory, Helsinki; and [20]Department of Oncology and Radiotherapy, Turku University Hospital, Turku, Finland

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

G. Manceau and S. Imbeaud contributed equally to this article.

Corresponding Author: Pierre Laurent-Puig, UMR-S775 Molecular basis of response to xenobiotics, 45 Rue des Saints-Pères, 75006 Paris, France. Phone: 331-4286-2081; Fax: 331-4286-2072; E-mail: pierre.laurent-puig@parisdescartes.fr

doi: 10.1158/1078-0432.CCR-13-2750

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.

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 treatments. Many studies have evaluated other potential biomarkers, typically using a candidate gene approach focused on different intracellular pathways downstream of EGFR or a genome-wide approach. At the present time, however, the routine use of these biomarkers is not warranted. Indeed, *BRAF* V600E mutation seems to be a marker of poor prognosis (9, 10, 14, 15). Furthermore, studies that examined *PIK3CA* mutations, loss of PTEN expression, *EGFR* gene copy number, and polymorphisms of fragment cγ receptors have reported conflicting results (14, 16–27). Finally, mRNA expression level of amphiregulin and epiregulin seems to be of limited 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.

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-FFPE) 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 5-fluorouracil–based regimen combined with irinotecan and oxaliplatin.

The last cohort of patients (validation set, VS) was comprised of patients from a randomized phase II trial sponsored by GERCOR (Groupe Cooperateur Multidisciplinaire en Oncologie). From the 65 patients eligible for this phase II study, 50 *KRAS* wild-type patients were considered for subsequent analysis. Among them, 45 patients had enough tumor material to be included (84%) with 26 having only formalin-fixed paraffin-embedded (FFPE) samples available, 6 with fresh-frozen (FF) samples only, and 13 having both FFPE and FF samples available. All patients in this cohort were treated with third-line therapy using a combination of irinotecan and panitumumab after a

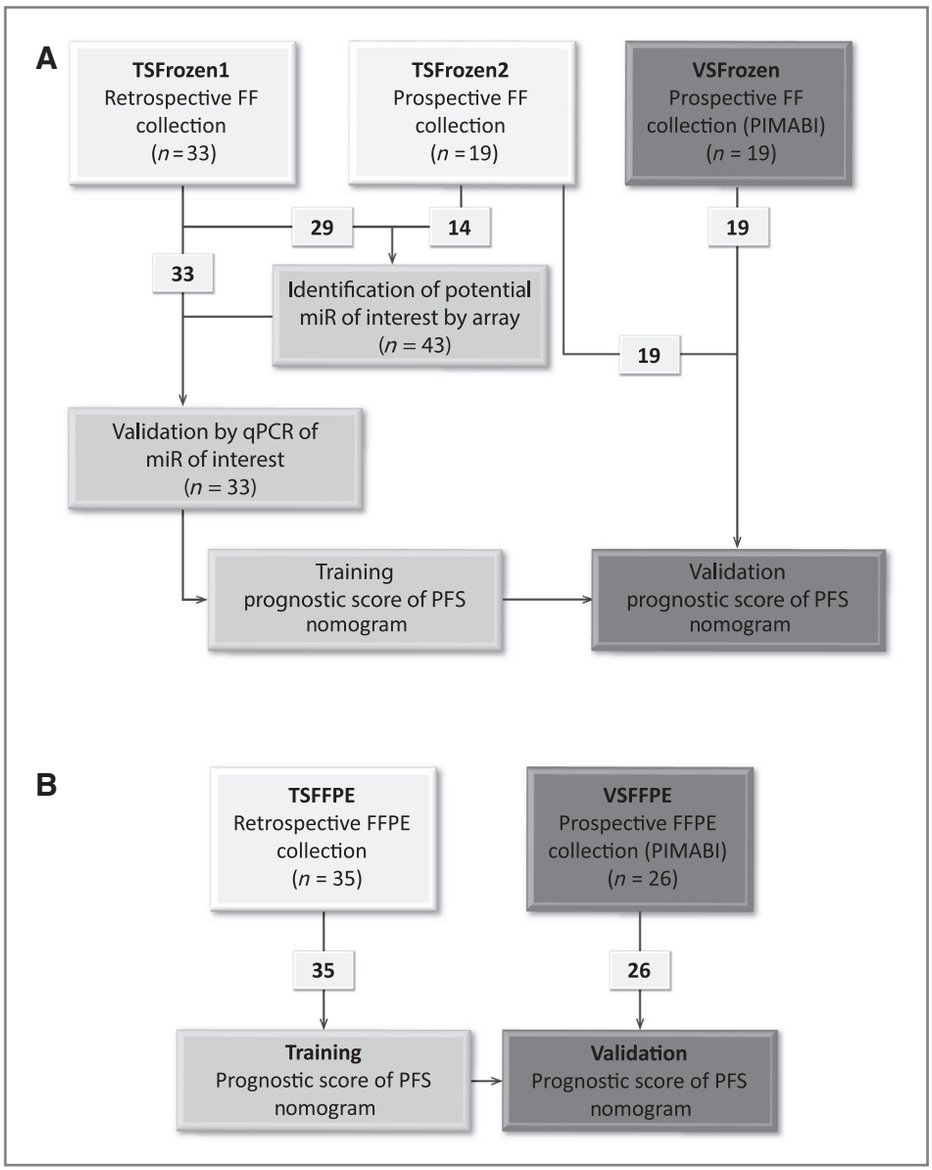

Figure 1. Flow chart.

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 $\log_2$-transformed. For expression analysis on TaqMan (Applied

Biosystems) of the 11 selected miRNAs, see Supplementary Methods.

Cell culture and transfection

Three colorectal adenocarcinoma cell lines (HTB-37, CCL-222, and CCL220-1) from the American Type Culture Collection (ATCC) were selected and used in the 3 month following reception of the cells for cell culture studies. The authentication of the cells has been provided by ATCC using the short tandem repeat profiling method. All cells were transfected with miRVanamiRNA (Ambion) mimic negative control or hsa-miR-31-3p miRVanamiRNA mimic (see Supplementary Methods).

In silico analysis

We developed a database integrating contemporary miRNA target predictions from six individual databases (PITA; picTar 5-way Targetscan microRNA.org; MicroCosmandmiRDB). These databases allowed for the determination of miRNAs cotargeted by candidate genes, taking into account the number of miRNA prediction databases that predicted each miRNA/target relationship and the rank of this prediction. This database was current as of November 2012, when our analysis was performed.

Statistical analyses

Prediction of survival risks used the miRNA expression data in a supervised principal component (SuperPC) approach analysis, a modification of the conventional perchloric acid as described by Bair and colleagues (35, 36). To evaluate the predictive value of the method, a leave-one-out cross-validation was performed and permutation analysis ($n = 100$) estimated the statistical significance of separation of the Kaplan–Meier survival curves. A prognostic index is provided for each patient with a log expression profile given a vector x; a high value of the prognostic index corresponding to a high value of hazard of death.

Survival statistical analysis was performed using the R packages "survival" and "rms." Univariate and multivariate analyses used a Cox proportional regression hazard model generating an HR and a performance index (area under the curve, AUC). Nomograms were developed on the basis of Cox proportional regression hazard models, which predict the probability of progression-free survival (PFS) based on a training set of patients. Internal validation relied on 150 bootstrap resamples, then independent validation. The validation set series and whole series (training + validation series) were submitted to a multivariate Cox model analysis, including the Eastern Cooperative Oncology Group (ECOG) performance status and the level of lactate dehydrogenase (LDH) when available. These two variables were obtained at the time of the first administration of anti-EGFR therapy. The analysis was performed by STATA version 11. The patients were dichotomized in two groups, high and low risk, as determined above. The ECOG performance status was obtained in 108 patients. The LDH have been prospectively recorded for the patients included in the phase II PIMABI study.

Gene and miRNA expression value comparison analyses were performed using the nonparametric test (Kruskal–Wallis tests) with the pairwise Wilcox test function in R using the stats library.

False-discovery rate–adjusted P values were calculated using the Benjamini and Hochberg procedure for multiple testing correction. The correction test function was used to calculate Pearson correlations between expression values together with matching P values. Statistical significance was set at $P < 0.05$ for all analyses.

Results

Hsa-miR-31-3p survival analysis

Patient characteristics of the different sets are described in Table 1. Overall, 132 patients with mCRC were included in this study. All patients were wild-type *KRAS* based on screening of exons 2, 3, and 4. From training sets 1 and 2, 43 RNAs were extracted from frozen tumor samples and analyzed by Illumina Beadchips. Using SuperPC analysis, univariate 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 TaqMan 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 AUC superior to 0.70, whatever the time threshold tested (Supplementary Fig. S3).

Table 1. Baseline patient characteristics

Patient data	Training set 1 ($N = 33$)	Training set 2 ($N = 19$)	Training set 3 ($N = 35$)	Validation set 1a ($N = 19$)	Validation set 1b ($N = 26$)
Gender					
Male	24 (72.7%)	11 (57.9%)	16 (45.7%)	12 (63.2%)	18 (69.2%)
Female	9 (27.3%)	8 (42.1%)	19 (54.3%)	7 (21.2%)	8 (30.8%)
Age					
Mean	55.6	65.0	63.3	61.8	60.2
Range	(22.0–78.1)	(47.3–77.9)	(37–82)	(33.9–84.5)	(34–82)
Chemotherapy regimen					
Cetuximab	0	0	2 (5.7%)	0	0
Cetuximab + irinotecan	24 (72.7%)	11 (57.9%)	16 (45.7%)	0	0
Cetuximab + Xeloda	1 (3.0%)	0	0	0	0
Cetuximab + FOLFIRI	5 (15.2%)	4 (21.1%)	4 (11.4%)	0	0
Cetuximab + FOLFOX	2 (6.0%)	0	1 (2.9%)	0	0
Panitumumab	0	4 (21.1%)	8 (22.9%)	0	0
Panitumumab + XELIRI	0	0	1 (2.9%)	0	0
Panitumumab + irinotecan	1 (3.0%)	0	3 (8.6%)	19 (100%)	26 (100%)
Number of treatment lines before anti-EGFR therapy					
Median	2	2	3	2	2
Range	(1–5)	(1–6)	(1–6)	(1–6)	(1–6)
Complete response	2 (6.0%)	0	0	1 (5.3%)	2 (7.7%)
Response according to RECIST criteria					
Partial response	10 (30.3%)	4 (21.1%)	13 (37.1%)	7 (36.8%)	8 (30.8%)
Stable disease	11 (33.3%)	9 (47.4%)	10 (28.6%)	6 (31.6%)	8 (30.8%)
Progressive disease	10 (30.3%)	6 (31.6%)	12 (34.3%)	5 (26.3%)	7 (26.9%)

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 2.79; 95% CI, 1.12–6.96; $P = 0.027$ was observed between the high- and low-risk group of patients. This threshold was then validated on VS-FFPE from the PIMABI trial for patients in which only FFPE samples were available. An HR for PFS of 5.1; 95% CI, 1.48–14.44; $P = 0.01$ was observed.

We then added patients from VS-Frozen for whom we also had FFPE samples ($n = 13$). The PFS HR between high- and low-risk patients was of 2.44; 95% CI, 1.1–5.4; $P = 0.028$. When we added 6 patients to this cohort for whom only FF tissues were available to test whole series of PIMABI, the PFS HR between high- and low-risk patients was of 2.46; 95% CI, 1.22–4.94; $P = 0.012$ (Fig. 4). Finally, we observed a significant inverse correlation between the percentage variation of the RECIST criteria and hsa-miR-31–3p expression ($r^2 = 0.49$; $P = 0.0035$). This result was confirmed by the significant association between patient risk status and the percentage of variation of RECIST criteria ($P = 0.02$; Kruskal–Wallis rank test, Fig. 3). Multivariate analyses were performed, including the BRAF status (i.e., mutated vs. nonmutated), the LDH level and the ECOG performance status at time of inclusion in PIMABI phase II study in *RAS* wild-type population. The status of patients determined by the level of expression of the hsa-miR-31-3p remains significant. The PFS HR and overall survival (OS) HR between high- and low-risk patients were 2.6; 95% CI, 1.1–5.8; $P = 0.023$ and 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; $P = 0.008$.

Hsa-miR-31-3p targets

Three colorectal cancer cell lines were transfected with hsa-miR-31-3p mimic or with a mimic control. The transfection efficacy was demonstrated by a 1,500 times average increase of hsa-miR-31-3p levels. Expression profile analysis of transfected cells allowed us to identify 47 genes

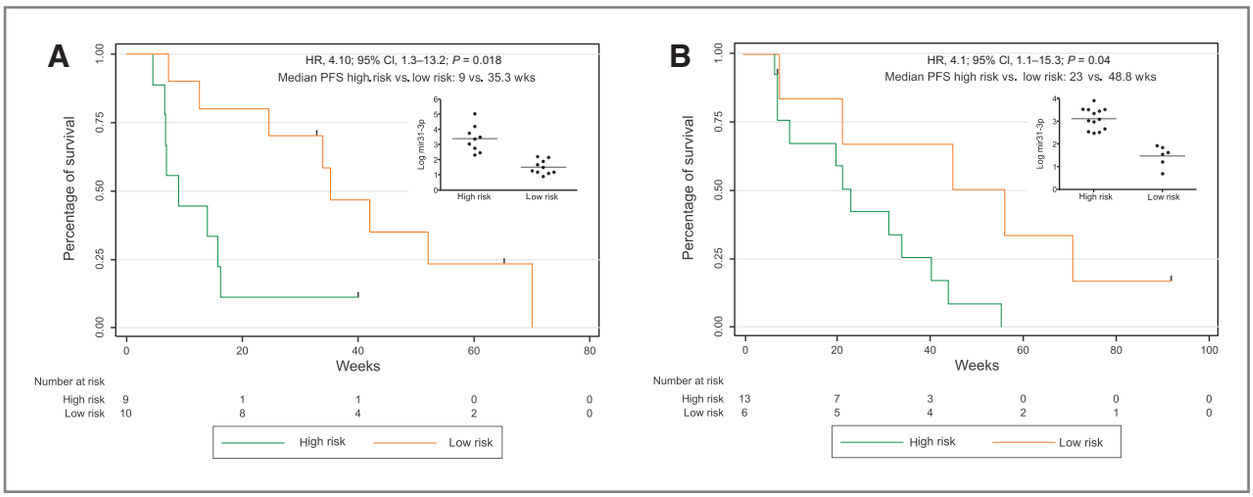

Figure 2. Kaplan–Meier curves for risk groups obtained from the second training set (A) and the validation set 1a (B).

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 permutation 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 miRNAs 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 refractory 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 independently confirm those of a Finnish group (32) who demonstrated 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

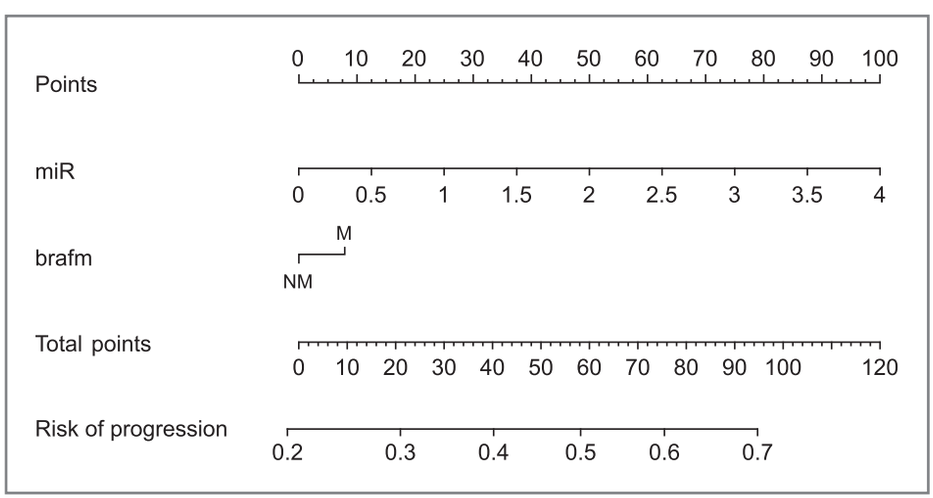

Figure 3. Nomogram to predict PFS survival using log-transformed hsa-miR-31-3p expression levels (miR) and the BRAF mutational status (brafm). To use the nomogram, locate patient's variable on the corresponding axis; draw a line to the points axis, sum the points, and draw a line from the total points axis to the PFS probability axis.

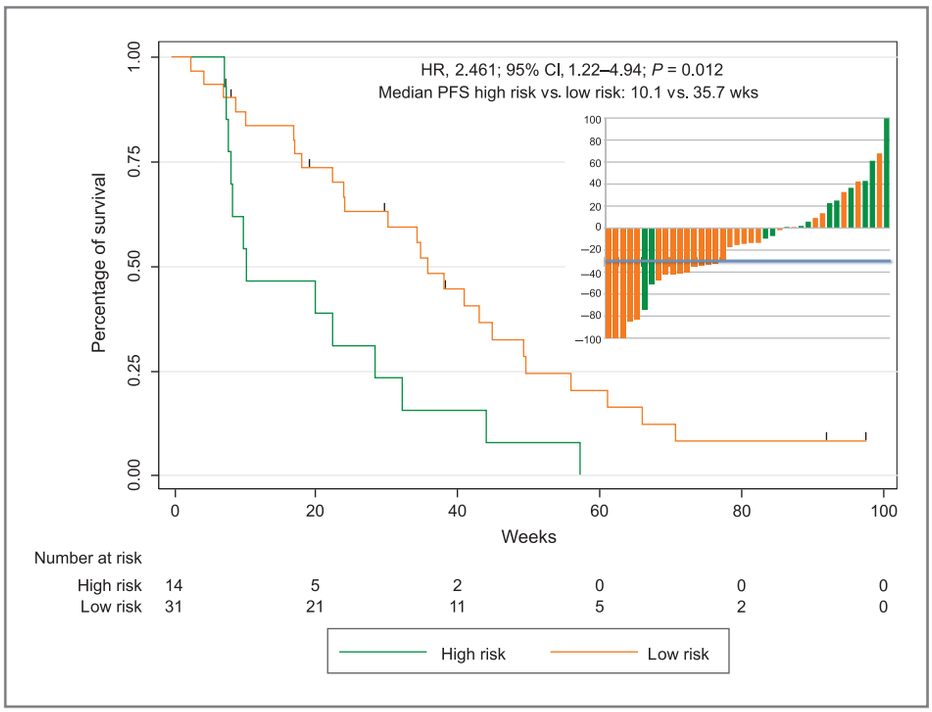

Figure 4. Kaplan–Meier PFS curves and the waterfall plot for RECIST criteria for the whole series of validation (PIMABI phase II; $n = 45$). Patients are classified in high and low risk of progression according to the value obtained for FFPE samples for patients with FFPE samples ($n = 39$) and for FF samples for those with only FF samples ($n = 6$). The RECIST criteria were available only for 40 patients.

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 directly 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

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 (44, 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. Thiébaut, F. Liébaert, 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 Amgen, Merck Serono, and Sanofi. S. Tejpar reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Merck Serono. P. Österlund 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

Conception and design: G. Manceau, R. Thiébaut, F. Liébaert, F. Rousseau, B. Génin, P. Österlund, S. Knuutila, P. Laurent-Puig

Development of methodology: S. Imbeaud, R. Thiébaut, 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.): R. Thiébaut, 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. Tejpar, N. Mosakhani, P. Österlund, R. Ristamäki, V. Sarhadi, S. Knuutila, V. Boige, T. André, P. Laurent-Puig

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Manceau, S. Imbeaud, R. Thiébaut, B. Génin, M. Vincent, F. Penault-Llorca, J.-L. Van Laethem, S. Tejpar, S. Rossi, N. Mosakhani, P. Österlund, S. Knuutila, T. André, P. Laurent-Puig

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

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

Study supervision: G. Manceau, R. Thiébaut, F. Liébaert, T. André, P. Laurent-Puig

Acknowledgments

The authors thank Céline Vazart and Virginie Decaulne for their expertise and contribution associated with the extraction and quantification of miRNA for this study.

Grant Support

This work was funded by the Programme Hospitalier de Recherche Clinique (PHRC), Institut National de la Santé et de la Recherché Médicale (INSERM) through the Biointelligence program, and by IntegraGen through the Biomos program funded by Banque Publique d'Investissement, France. PETACC3 research on miRNAs is supported by the Foundation Fournier-Majoie, FFMI, Belgium. S. Tejpar is a senior clinical investigator of the Fund for Scientific Research Flanders and is supported by Belgian National Cancer Plan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 29, 2013; revised March 14, 2014; accepted April 8, 2014; published OnlineFirst April 25, 2014.

References

1. van der Pool AE, Damhuis RA, Ijzermans JN, de Wilt JH, Eggermont AM, Kranse R, et al. Trends in incidence, treatment and survival of patients with stage IV colorectal cancer: a population-based series. Colorectal Dis 2012;14:56–61.
2. Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 2011;39:D945–50.
3. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:1626–34.
4. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.
5. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 2009;360:563–72.
6. Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, et al. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. J Clin Oncol 2010;28:4706–13.
7. Ocvirk J, Brodowicz T, Wrba F, Ciuleanu TE, Kurteva G, Beslija S, et al. Cetuximab plus FOLFOX6 or FOLFIRI in metastatic colorectal cancer: CECOG trial. World J Gastroenterol 2010;16:3133–43.
8. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated

metastatic colorectal cancer: the PRIME study. J Clin Oncol 2010; 28:4697–705.
9. Bokemeyer C, Bondarenko I, Hartmann JT, de Braud F, Schuch G, Zubel A, et al. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. Ann Oncol 2011;22:1535–46.
10. Van Cutsem E, Kohne CH, Lang I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol 2011;29:2011–9.
11. Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 2009;27:2091–6.
12. Van Cutsem E, Dicato M, Arber N, Berlin J, Cervantes A, Ciardiello F, et al. Molecular markers and biological targeted therapies in metastatic colorectal cancer: expert opinion and recommendations derived from the 11th ESMO/World Congress on Gastrointestinal Cancer, Barcelona, 2009. Ann Oncol 2010;21 Suppl 6:vi1–10.
13. Lievre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol 2008;26:374–9.
14. Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Borger ME, et al. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. Eur J Cancer 2010;46:1997–2009.
15. Bokemeyer C, Cutsem EV, Rougier P, Ciardiello F, Heeger S, Schlichting M, et al. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. Eur J Cancer 2012;48:1466–75.
16. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006;66:3992–5.
17. Prenen H, De Schutter J, Jacobs B, De Roock W, Biesmans B, Claes B, et al. PIK3CA mutations are not a major determinant of resistance to the epidermal growth factor receptor inhibitor cetuximab in metastatic colorectal cancer. Clin Cancer Res 2009;15:3184–8.
18. Sartore-Bianchi A, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S, et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 2009;69:1851–7.
19. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010;11:753–62.
20. Laurent-Puig P, Cayre A, Manceau G, Buc E, Bachet JB, Lecomte T, et al. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. J Clin Oncol 2009;27:5924–30.
21. Frattini M, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, et al. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. Br J Cancer 2007;97: 1139–45.
22. Loupakis F, Pollina L, Stasi I, Ruzzo A, Scartozzi M, Santini D, et al. PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. J Clin Oncol 2009;27:2622–9.
23. Sartore-Bianchi A, Moroni M, Veronese S, Carnaghi C, Bajetta E, Luppi G, et al. Epidermal growth factor receptor gene copy number and clinical outcome of metastatic colorectal cancer treated with panitumumab. J Clin Oncol 2007;25:3238–45.
24. Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, et al. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. Lancet Oncol 2005;6:279–86.
25. Italiano A, Follana P, Caroli FX, Badetti JL, Benchimol D, Garnier G, et al. Cetuximab shows activity in colorectal cancer patients with tumors for which FISH analysis does not detect an increase in EGFR gene copy number. Ann Surg Oncol 2008;15:649–54.
26. Bibeau F, Lopez-Crapez E, Di Fiore F, Thezenas S, Ychou M, Blanchard F, et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122–9.
27. Park SJ, Hong YS, Lee JL, Ryu MH, Chang HM, Kim KP, et al. Genetic polymorphisms of FcgammaRIIa and FcgammaRIIIa are not predictive of clinical outcomes after cetuximab plus irinotecan chemotherapy in patients with metastatic colorectal cancer. Oncology 2012;82:83–9.
28. Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 2007;25:3230–7.
29. Jacobs B, De Roock W, Piessevaux H, Van Oirbeek R, Biesmans B, De Schutter J, et al. Amphiregulin and epiregulin mRNA expression in primary tumors predicts outcome in metastatic colorectal cancer treated with cetuximab. J Clin Oncol 2009;27:5068–74.
30. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol 2009;27:5848–56.
31. Corte H, Manceau G, Blons H, Laurent-Puig P. MicroRNA and colorectal cancer. Dig Liver Dis 2012;44:195–200.
32. Mosakhani N, Lahti L, Borze I, Karjalainen-Lindsberg ML, Sundstrom J, Ristamaki R, et al. MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF. Cancer Genet 2012;205: 545–51.
33. Andre T, Blons H, Mabro M, Chibaudel B, Bachet JB, Tournigand C, et al. Panitumumab combined with irinotecan for patients with KRAS wild-type metastatic colorectal cancer refractory to standard chemotherapy: a GERCOR efficacy, tolerance, and translational molecular study. Ann Oncol 2013;24:412–9.
34. Didelot A, Le Corre D, Luscan A, Cazes A, Pallier K, Emile JF, et al. Competitive allele specific TaqMan PCR for KRAS, BRAF and EGFR mutation detection in clinical formalin-fixed paraffin-embedded samples. Exp Mol Pathol 2012;92:275–80.
35. Bair E, Hastie T, Paul D, Tibshirani R. Prediction by supervised principal components. J Am Stast Ass 2006;101:119–37.
36. Bair E, Tibshirani R. Semi-supervised methods to predict patient survival from gene expression data. PLoS Biol 2004;2:E108.
37. Wei J, Gao W, Zhu CJ, Liu YQ, Mei Z, Cheng T, et al. Identification of plasma microRNA-21 as a biomarker for early detection and chemosensitivity of non–small cell lung cancer. Chin J Cancer 2011;30: 407–14.
38. Odenthal M, Bollschweiler E, Grimminger PP, Schroder W, Brabender J, Drebber U, et al. MicroRNA profiling in locally advanced esophageal cancer indicates a high potential of miR-192 in prediction of multimodality therapy response. Int J Cancer 2013;133: 2454–63.
39. Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, et al. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. Cancer Res 2010;70:3606–17.
40. Pichler M, Winter E, Stotz M, Eberhard K, Samonigg H, Lax S, et al. Downregulation of KRAS-interacting miRNA-143 predicts poor prognosis but not response to EGFR-targeted agents in colorectal cancer. Br J Cancer 2012;106:1826–32.
41. Borralho PM, Kren BT, Castro RE, da Silva IB, Steer CJ, Rodrigues CM. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. FEBS J 2009;276: 6689–700.
42. Shen J, Xia W, Khotskaya YB, Huo L, Nakanishi K, Lim SO, et al. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature 2013;497:383–7.

43. Franovic A, Gunaratnam L, Smith K, Robert I, Patten D, Lee S. Translational upregulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc Natl Acad Sci U S A 2007;104:13092–7.
44. Van Cutsem E, Labianca R, Bodoky G, Barone C, Aranda E, Nordlinger B, et al. Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3. J Clin Oncol 2009;27:3117–25.
45. Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60–00 trial. J Clin Oncol 2010;28:466–74.