

MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF

Neda Mosakhani ^a, Leo Lahti ^b, Ioana Borze ^a,
Marja-Liisa Karjalainen-Lindsberg ^a, Jari Sundström ^c,
Raija Ristamäki ^d, Pia Österlund ^e, Sakari Knuutila ^{a,*}, Virinder Kaur Sarhadi ^a

^a Department of Pathology, Haartman Institute and HUSLAB, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland; ^b Laboratory of Microbiology, Wageningen University, the Netherlands; ^c Department of Pathology, University of Turku and Turku University Central Hospital, Turku, Finland; ^d Department of Oncology and Radiotherapy of University Hospital of Turku, Turku, Finland; ^e Department of Oncology of Helsinki University Central Hospital and University of Helsinki, Helsinki, Finland

Anti-EGFR monoclonal antibodies (anti-EGFRmAb) serve in the treatment of metastatic colorectal cancer (mCRC), but patients with a mutation in KRAS/BRAF and nearly one-half of those without the mutation fail to respond. We performed microRNA (miRNA) analysis to find miRNAs predicting anti-EGFRmAb efficacy. Of the 99 mCRC patients, we studied differential miRNA expression by microarrays from primary tumors of 33 patients who had wild-type KRAS/BRAF and third- to sixth-line anti-EGFRmAb treatment, with/without irinotecan. We tested the association of each miRNA with overall survival (OS) by the Cox proportional hazards regression model. Significant *miR-31** up-regulation and *miR-592* down-regulation appeared in progressive disease versus disease control. *miR-31** expression and down-regulation of its target genes *SLC26A3* and *ATN1* were verified by quantitative reverse transcriptase polymerase chain reaction. Clustering of patients based on miRNA expression revealed a significant difference in OS between patient clusters. Members of the *let-7* family showed significant up-regulation in the patient cluster with poor OS. Additionally, *miR-140-5p* up-regulation and *miR-1224-5p* down-regulation were significantly associated with poor OS in both cluster analysis and the Cox proportional hazards regression model. In mCRC patients with wild-type KRAS/BRAF, miRNA profiling can efficiently predict the benefits of anti-EGFRmAb treatment. Larger series of patients are necessary for application of these miRNAs as predictive/prognostic markers.

Keywords Colorectal cancer, miRNA, anti-EGFR treatment, wild-type KRAS, wild-type BRAF

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Colorectal carcinoma (CRC) is the third most common human malignant disease worldwide. Molecularly targeted therapies have markedly changed the strategies used in CRC treatment. Recent results from phase II and III clinical trials demonstrate that patients with metastatic colorectal cancer (mCRC) benefit from therapy with monoclonal antibodies directed against EGFR (anti-EGFR monoclonal

antibodies [anti-EGFRmAb]), when used either as monotherapy or combined with chemotherapy (1); however, only 10–20% of chemorefractory mCRC patients respond to anti-EGFRmAb as a single agent (2,3). The mCRC patients with KRAS and BRAF mutations in their tumor cells who do not benefit from the anti-EGFRmAb treatment comprise up to 55% of non-responders (4,5). Moreover, nearly one-half of the patients without KRAS and BRAF mutations still show no benefit from the antibody treatment, and recent findings also indicate that some chemotherapy combinations may lack any anti-EGFRmAb efficacy (4,6,7). Therapeutically, it is highly important to understand the molecular mechanisms behind the drug resistance in this patient group.

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* Corresponding author.

E-mail address: sakari.knuutila@helsinki.fi

Recently, microRNAs (miRNAs) have been implicated in many biological and tumor-related processes such as progression, invasion, and oncogenesis. Numerous studies have shown that miRNA profiling may distinguish tumors according to their prognostic and predictive properties (8,9). Moreover, miRNAs are indirect, potent regulators of drug efficacy and of pharmacogenomic markers, because many important drug-related genes are targeted by miRNAs (10). Studies have recently focused on the significance of miRNAs in CRC (11–13). No report, however, has discovered miRNA profiling in CRC patients with wild-type *KRAS* and *BRAF* in relation to their response to anti-EGFRmAb treatment. We therefore performed miRNA analyses to observe differences in miRNA profiles between groups of chemorefractory mCRC patients, ones resistant and non-resistant to anti-EGFRmAb treatment, with or without irinotecan, and to identify miRNAs related to therapeutic response.

Materials and methods

Patient selection

We selected 99 patients with mCRC treated at Helsinki University Central Hospital and at Turku University Central Hospital. We included, however, only those 33 patients for the final analysis 1) for whom tissue was available from the primary tumor at diagnosis before the start of any treatment; 2) who had wild-type *KRAS* and *BRAF* status; and 3) who had had third- to sixth-line treatment with cetuximab or panitumab with or without irinotecan, and were chemorefractory or intolerant to irinotecan, oxaliplatin, and 5-fluorouracil; and 4) who had in their history no other malignancy. These included 21 men and 12 women, with a median age of 62 years (range 37–83). The site of the primary tumor was the colon in 26 patients and the rectum in 7.

Each patient's response to anti-EGFRmAb treatment was assessed using the Response Evaluation Criteria in Solid Tumors, version 1.0 (14). Patients who responded to treatment or had stable disease were considered as the disease control (DC) group, and the remaining patients were classified as the progressive disease (PD) group. The tumor content of each sample was determined by pathologists at the Helsinki and Turku University Central Hospitals. The control RNA sample was commercially available from colon tissue (FirstChoice Human Total RNA, Applied Biosystems/Ambion, Austin, TX). The study was approved by the HUS (Hospital district of Helsinki and Uusimaa) ethics committee, as no: 173/13/03/02/09.

Mutation analysis of *KRAS* and *BRAF*

DNA extraction from tumor tissue samples was performed with the QIAamp DNA FFPE Mini Kit (Qiagen, Valencia, CA). The TheraScreen *KRAS* Mutation Kit (Qiagen, DxS, Manchester, UK) was used for the detection of seven *KRAS* mutations in codons 12 and 13, and the *BRAF* Mutation Test Kit (Qiagen, DxS, Manchester, UK) for the detection of the V600E mutation in *BRAF*. The *BRAF* mutation test was performed only in wild-type *KRAS* patients because, according to several reports, *KRAS* and *BRAF* mutations in CRCs tumors are nearly mutually exclusive (15). Patients

with *BRAF* and *KRAS* mutations were excluded from further analysis.

RNA extraction

Total RNA, including miRNA, was extracted with the miRNeasy FFPE Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) was used for the quantification of RNA, and Agilent's Bioanalyzer for checking the quality of total RNA with the RNA 6000 chip and miRNA with the small RNA chip (Agilent Technologies, Santa Clara, CA).

miRNA microarray

Agilent's miRNA Microarray System V2 (723 human and 76 human viral miRNAs, Sanger database v.10.1; <http://microrna.sanger.ac.uk>) and miRNA Complete Labeling and Hybridization Kit Protocol version 2.0 (Agilent Technologies) was used for processing the samples, as described earlier (16). Briefly, 100 ng of total RNA was dephosphorylated with calf intestine phosphatase at 37°C for 30 minutes; 100% DMSO (dimethyl sulfoxide) was used for denaturation at 100°C for 5 minutes. Samples were labeled with cyanine 3-pCp, vacuum-dried at medium heat, and resuspended in nuclease-free water. The samples were hybridized on the Agilent Human miRNA Microarrays, washed with washing buffers, and scanned with Agilent's Scanner (model G2539A). The raw data were reprocessed with Agilent's Feature Extraction v. 9.5 software.

Statistical methods

Analysis of miRNA and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) data

Statistical analysis of microarray data was performed with GeneSpring GX Analysis v.11.0.2 software (Agilent). The data were preprocessed by performing a normalization between all arrays using the 75th percentile method, and taking the log₂ transformation. The miRNAs not detected in any of the samples or controls were removed from the analysis. The significance of differential expression between two groups of samples was estimated by *t* test. The false discovery rate (FDR) method of Benjamini-Hochberg served to correct for multiple testing. miRNAs with a fold-change (FC) of >1.5 were considered differentially expressed. For qRT-PCR, the statistical significance of differences in miRNA expression in the pair-wise comparisons was calculated with the Student *t* test, with a *P* < 0.05 significance threshold.

Survival analysis

The time of diagnosis of mCRC and death was considered overall survival with mCRC (OS with mCRC). The start of anti-EGFRmAb treatment until death was considered to calculate OS with anti-EGFRmAb therapy (OS with anti-EGFRmAb). First, we applied unsupervised clustering (K-means with two clusters and Euclidean distance) on miRNA microarray data to cluster the patients into two groups based on their miRNA profiles. Then, Kaplan-Meier survival analyses were performed on these patient groups,

and the differences in OS between the groups were compared by the Wilcoxon test. Next, we identified a set of miRNAs where the expression was significantly different between the two patient groups.

We also studied the association of each miRNA individually with OS using the Cox proportional hazards regression model. For miRNAs that also showed association with OS in clustering analysis and *let-7f*, we calculated the likelihood of significant positive or negative correlation of miRNA expression with survival, using Pearson correlation coefficients (r) ($P < 0.05$).

Verification of microarray results with qRT-PCR

We selected *miR-31**, *miR-592*, and *let-7f* for verification by qRT-PCR. The miScript Reverse Transcription Kit (Qiagen, Valencia, CA) was used for reverse transcription of RNA, according to the manufacturer's guidelines. qRT-PCR was performed on a LightCycler v.3.5 software (Roche Applied Science, Mannheim, Germany) by the miScript SYBR Green PCR Kit (Qiagen). Each reaction was performed in a 20 μ l volume with 5 ng of template cDNA. The primers for amplification of selected miRNAs and small nuclear (sn) RNA U6 were commercially available (Qiagen). PCR for each RNA sample was performed in duplicate, and every run included a control without a template. The U6 primer assay (Qiagen) served as an endogenous control for normalization. The relative quantification for each miRNA, compared with U6, was calculated using the equation $2^{-\Delta\Delta Ct}$.

Predicted targets of differentially expressed miRNAs and pathway analysis

To identify predicted mRNA targets for the differentially expressed miRNAs, we used six target prediction databases: miRBase (<http://microrna.sanger.ac.uk>), TargetScan (<http://www.targetscan.org>), miRanda (<http://www.microRNA.org>), mirTarget2 (<http://mirdb.org/miRDB>), Tarbase (<http://diana.cslab.ece.ntua.gr/tarbase>), and PICTAR (<http://pictar.mdc-berlin.de/>). Predicted mRNA targets of the differentially expressed miRNAs were screened by Chipster v1.4.7 (<http://chipster.csc.fi/>) for significant enrichment of Kyoto Encyclopedia of Genes and Genomics (KEGG) pathways and over-represented pathways in the ConsensusPathDB (CPDB; <http://cpdb.molgen.mpg.de>) by the hypergeometric test ($P < 0.05$).

Evaluation of mRNA levels of *SLC26A3* and *ATN1* with qRT-PCR

We selected *SLC26A3* and *ATN1* to evaluate their mRNA levels in PD and DC groups, as both were predicted target genes of *miR-31**, which was differentially expressed between PD and DC—and these genes are reportedly related to drug response in CRC. Following the manufacturer's guidelines, 100 ng of RNA was reverse-transcribed into cDNA with an RT² First Strand Kit (Qiagen). We performed qRT-PCR with the RT² SYBR Green PCR Master Mix (Qiagen) on a LightCycler v.3.5 software (Roche Applied Science). Each reaction was performed in a 20 μ l volume with a 10 ng cDNA synthesis reaction. Human 18S ribosomal RNA (rRNA),

SLC26A3, and *ATN1* primers were purchased from Qiagen. Human 18S rRNA served as a housekeeping gene for normalization. Cycling conditions for qRT-PCR consisted of an initial incubation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each RNA sample was performed at least in duplicate, and every run included a control without a template. To calculate gene expression, we used the $2^{-\Delta\Delta Ct}$ comparative method.

Results

Response to anti-EGFRmAb in wild-type *KRAS* and *BRAF* patients

Among CRC tumors with wild-type *KRAS* and *BRAF*, 33 of them had criteria meriting further analysis. Objective best responses for 33 patients were as follows: partial response in 8 patients, long (≥ 3 months) stable disease in 11, and progressive disease in 14. The PD group included 11 men and 3 women, with a median age of 59.5 years (range 37–69). The site of the primary tumor in the PD group was the colon in 11 patients and the rectum in 3. Ten men and 9 women of median age 66 years (range 37–76) were in the DC group; the site of the primary tumor in 15 of these patients was the colon and in 4 was the rectum.

miRNA expression profile of disease control and progressive disease groups

We found two miRNAs, up-regulated *miR-31** ($P \leq 0.04$, FC ≥ 2.1), and down-regulated *miR-592* ($P \leq 0.04$, FC ≥ 1.9), which showed a significant difference in PD compared with DC, although no miRNA appeared differentially expressed with a $q < 0.2$ cut-off. qRT-PCR showed a similar significant differential expression of *miR-31** as with the microarray analysis.

Correlation of miRNA expression with OS in mCRC

Unsupervised clustering and division of the patients into two groups (groups 1 and 2; **Figure 1A**) based on their miRNA expression showed significant differences in OS with mCRC (4.23 years for group 1 vs. 2.89 for group 2; $P \leq 0.03$) between the two groups by Kaplan-Meier analysis (**Figure 1B**). Comparison of miRNA expression in these 2 patient groups revealed 75 differentially expressed miRNAs, which were expressed in all the cases in at least 1 of the groups and had $q < 0.0001$. A significant up-regulation of 58 miRNAs and down-regulation of 17 miRNAs appeared in the group of patients with poorer prognosis versus good prognosis (**Table 1**). Of these, up-regulation of the *let-7* family, including *let-7f*, *let-7g*, *let-7d*, *let-7i*, *let-7a*, *let-7e*, and *let-7b*, was evident in the group with poorer prognosis. Further, the differential expression of *let-7f* between the two groups was validated by qRT-PCR. Pearson correlation analysis of the qRT-PCR data of *let-7f* showed a similar negative correlation between *let-7f* expression and overall survival time but was not statistically significant ($R \leq -0.3$, $P \leq 0.08$). Based on the Cox proportional hazards regression model used to investigate the association of each

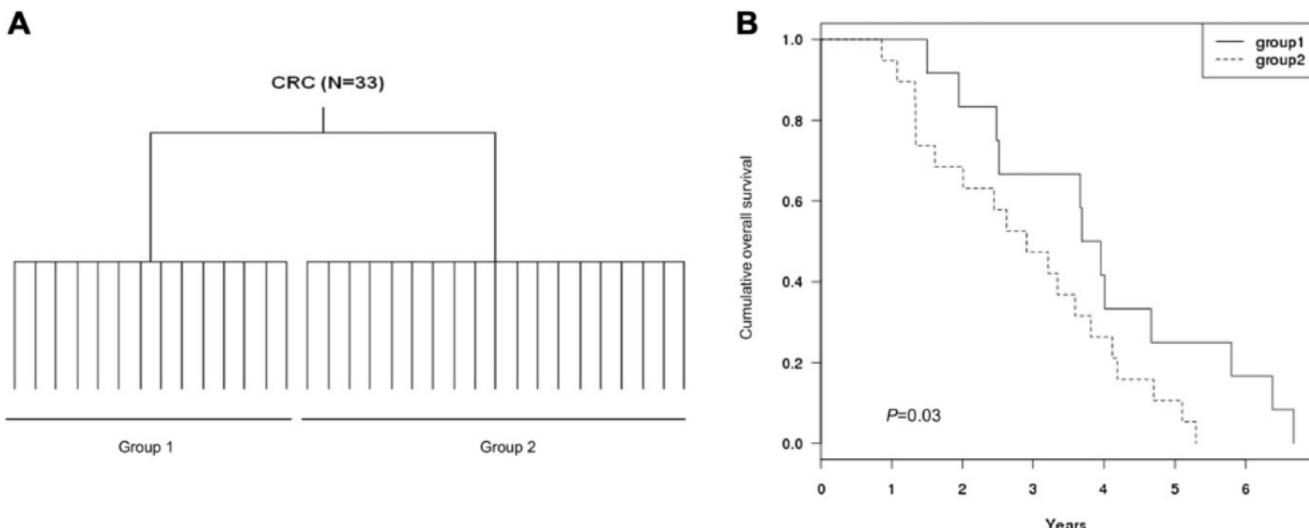


Figure 1 Clustering of CRC patients and their survival analysis. (A) Patients were divided into two clusters based on miRNA expression by 2K-mean clustering. (B) Survival analysis by Kaplan-Meier shows that group 2 had poorer prognosis and shorter overall survival (2.89 years) than group 1 (4.23 years).

miRNA expression individually with OS, we found expression levels of 11 miRNAs to be significantly correlated with OS ($P < 0.01$, $q \geq 0.3$) (Table 2). Two miRNAs, *miR-140-5p* (up-regulated in the patient cluster with shorter survival) and *miR-1224-5p* (down-regulated in the patient group with poorer prognosis), were common in both analyses.

Pearson correlation coefficient calculations showed that expression of *miR-1224-5p* had a significant positive correlation ($R \geq 0.4$, $P \leq 0.02$), and *miR-140-5p* had a significant negative correlation with OS with mCRC ($R \leq -0.4$, $P \leq 0.03$).

Validation of miRNA expression by qRT-PCR

Results of qRT-PCR were consistent with the results of the miRNA array. A significantly higher expression of *miR-31** ($P < 0.01$) was detectable in the PD group versus DC. Expression of *let-7f* was also significantly increased in the cluster of the patients with poorer prognosis ($P < 0.005$). Although *miR-592* showed a trend toward a decrease in expression level in the PD group versus the DC group, similar to the microarray results, this was not significant.

miRNAs targets and biological pathway

For *miR-31** and *miR-592*, which were differentially expressed in the PD versus DC groups, we identified, respectively, 557 and 35 predicted targets. Further, pathway enrichment analysis of the predicted *miR-31** and *miR-592* target genes showed seven significantly enriched pathways in PD compared with DC (Supplementary Table 1).

mRNA expression of *SLC26A3* and *ATN1*

The qRT-PCR analyses performed to study the expression of the genes targeted by *miR-31** revealed lower mRNA levels of *SLC26A3* and *ATN1* in the PD group than in the DC group.

We observed an 11-fold decrease in the *SLC26A3* mRNA level and a 4.6-fold decrease in the *ATN1* mRNA level in the PD group compared with the levels in the DC group.

Discussion

miRNAs as predictive biomarkers

In our miRNA expression study on primary untreated tumors of mCRC patients with wild-type *KRAS* and *BRAF*, we identified a signature associated with the efficacy of anti-EGFRmAb therapy. Up-regulation of *miR-31** and down-regulation of *miR-592* were associated with poor response to treatment. Moreover, expression of potential mRNA targets of *miR-31**, *ATN1*, and *SLC26A3* were lower in the PD group than in the DC group. Although very little is known regarding the function of *miR-31**, lower expression of *ATN1*, its potential mRNA target, is reported in chemoradiation-resistant colorectal cell lines (17). Furthermore, association of *SLC26A3* expression level with disease control and progression-free survival in *KRAS* wild-type CRCs is reported, and it is one of the four genes in the classification model for prediction of cetuximab efficacy (18). *SLC26A3* encodes an intestinal chloride ion transporter and is down-regulated in CRC, which suggests that *SLC26A3* may function as a tumor-suppressor gene (19). Higher level of expression of *miR-592* is reported in CRCs with proficient mismatch repair (pMMR) compared with CRCs with deficient mismatch repair (dMMR) (20), and dMMR cells are resistant to some anti-metabolites, such as 5FU, and to some platinum compounds, such as cisplatin and carboplatin (21,22).

Identification of predictive biomarkers with the aim to possibly individualize treatment is important, because treatments are expensive and lead to side effects. To our knowledge, our results are the first data suggesting that in the absence of *KRAS* and *BRAF* mutations, an increased *miR-31** and decreased *miR-592* expression are relevant in the development of predictive biomarkers for anti-EGFR

Table 1 Differentially expressed miRNAs between two clusters of patients^a

	miRNA	Fold Change
Up-regulated	<i>let-7f</i>	26.7
	<i>let-7a</i>	23.1
	<i>miR-199a-3p</i>	21.5
	<i>miR-26b</i>	19.3
	<i>let-7g</i>	16.8
	<i>miR-29b</i>	14.7
	<i>miR-20a</i>	13.3
	<i>miR-142-3p</i>	12.6
	<i>miR-424</i>	12.4
	<i>let-7d</i>	12.0
	<i>miR-148a</i>	11.9
	<i>let-7e</i>	11.5
	<i>miR-195</i>	11.4
	<i>miR-15b</i>	10.7
	<i>miR-107</i>	10.3
	<i>miR-30b</i>	10.1
	<i>miR-151-5p</i>	9.9
	<i>miR-19b</i>	9.9
	<i>miR-199b-5p</i>	9.7
	<i>miR-17</i>	9.7
	<i>miR-130a</i>	9.6
	<i>miR-374a</i>	9.5
	<i>miR-103</i>	9.3
	<i>miR-19a</i>	9.2
	<i>miR-199a-5p</i>	9.1
	<i>let-7i</i>	8.8
	<i>miR-146b-5p</i>	8.6
	<i>miR-451</i>	8.6
	<i>miR-101</i>	8.5
	<i>miR-20b</i>	8.4
	<i>miR-23a</i>	8.2
	<i>miR-215</i>	8.1
	<i>miR-21</i>	8.1
	<i>miR-106b</i>	7.9
	<i>miR-29a</i>	7.9
	<i>miR-140-5p</i>	7.2
	<i>miR-192</i>	7.1
	<i>miR-98</i>	6.9
	<i>miR-27b</i>	6.8
	<i>miR-126</i>	6.7
	<i>miR-28-5p</i>	6.7
	<i>miR-660</i>	6.4
	<i>miR-30c</i>	6.3
	<i>miR-16</i>	6.2
	<i>miR-10a</i>	6.2
	<i>miR-15a</i>	6.2
	<i>miR-93</i>	6.0
	<i>miR-27a</i>	5.8
	<i>miR-200a</i>	5.7
	<i>miR-10b</i>	5.7
	<i>miR-146a</i>	5.7
	<i>miR-194</i>	5.3
	<i>miR-34a</i>	4.6
	<i>miR-200b</i>	4.2
	<i>miR-425</i>	3.9
	<i>miR-429</i>	3.8
	<i>miR-25</i>	3.2
	<i>miR-362-5p</i>	3.1

Table 1 (continued)

	miRNA	Fold Change
Down-regulated	<i>miR-671-5p</i>	6.3
	<i>miR-188-5p</i>	5.9
	<i>miR-296-5p</i>	5.6
	<i>miR-1228</i>	5.5
	<i>miR-125a-3p</i>	5.4
	<i>miR-630</i>	5.1
	<i>miR-1234</i>	5.0
	<i>miR-1238</i>	4.6
	<i>miR-1225-3p</i>	4.5
	<i>miR-1224-5p</i>	4.4
	<i>miR-638</i>	4.0
	<i>miR-1225-5p</i>	4.0
	<i>miR-939</i>	3.8
	<i>miR-575</i>	3.8
	<i>miR-623</i>	3.7
	<i>miR-150</i>	3.3
	<i>miR-766</i>	2.7

^a Up- and down-regulated miRNAs in patients with poorer prognosis ($P < 0.0001$, $q < 0.0001$).

therapy in primary untreated tumors of mCRC and have important implications for the clinical management of CRC patients.

miRNAs as prognostic biomarkers

In addition to the miRNA expression profile associated with anti-EGFR therapy, we also found prognostic biomarkers based on the miRNA expression profile of primary tumors of mCRC patients with wild-type *KRAS* and *BRAF*. Patients clustered into two groups based on miRNA expression and showed significant differences in OS metastatic disease. Among the miRNAs associated with poor OS was *let-7f*, a member of the *let-7* miRNA family.

miRNAs of the *let-7* family are considered to be tumor suppressors, and their down-regulation occurs in numerous cancer types when compared to normal tissue. A higher level of *let-7*, associated with shorter survival in our study, is contradictory to the tumor-suppressor role of *let-7*. On the other hand, our results are similar to recent results on plasma miRNA levels in patients with cancers, such as lung cancer, myelodysplastic syndrome, and pancreatic cancer, which report a higher level of *let-7* as related to poor survival (23–25).

Other miRNAs found to be associated with poor OS in both cluster analysis and the Cox proportional hazards regression model were up-regulated *miR-140-3p* and down-regulated *miR-1224-5p*. *miR-140* is down-regulated in colon tumor tissue compared with adjacent normal tissue, but is up-regulated in colon cancer stem-like cells. Its expression is related to chemoresistance, and blocking it partially sensitizes colon cancer stem-like cells that are resistant to 5-FU treatment (26). Elevated levels are also reported upon malignant progression of gliomas (27). A decreased level of *IGFBP-5*, an *miR-140* target (28), is related to disease recurrence in lung cancer (29) and to tamoxifen resistance in breast cancer (30).

Table 2 Eleven miRNAs showing a significant correlation between their expression levels and survival or progression ($P < 0.01$)

OS	miRNA
Diagnosis-Death ^a	<i>miR-765, miR-1224-5p, miR-671-5p, miR-145, miR-328, miR-143*, miR-337-3p, miR-140-5p, miR-338-3p, miR-637</i>
Anti-EGFRmAb-Death ^b	<i>miR-637</i>

^a Time of diagnosis until death.^b Start of anti- EGFRmAb therapy until death.

Epigenetic silencing of *miR-1224* by hypermethylation is independently associated with tumor progression in bladder cancer (31). *miR-1224* is a negative regulator of *TNF-a* (32), and its down-regulation could thus theoretically lead to an increased *TNF-a* level, and subsequently could stimulate NF- κ B signaling. nuclear factor of kappa (NF-B) activation in primary tumors of mCRC patients is associated with shorter OS (33), and NF- κ B protein expression is reported to predict response and survival in mCRC treated with cetuximab and irinotecan therapy (34).

Our findings suggest that miRNA profiling of primary tumors has prognostic value for the OS and predicts benefits for mCRC patients who previously had third- to sixth-line treatment with anti-EGFR-based therapy. However, larger sample sizes and further independent studies are necessary to assess the sensitivity and specificity of the predictions and to validate the application of these miRNAs as future prognostic and predictive markers for chemorefractory mCRC patients in advanced stages of cancer.

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Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.cancergen.2012.08.003>.

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