Molecular Medicine in Practice

Specific Alterations of MicroRNA Transcriptome and Global Network Structure in Colorectal Carcinoma after Cetuximab Treatment

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Abstract

The relationship between therapeutic response and modifications of microRNA (miRNA) transcriptome in colorectal cancer (CRC) remains unknown. We investigated this issue by profiling the expression of 667 miRNAs in 2 human CRC cell lines, one sensitive and the other resistant to cetuximab (Caco-2 and HCT-116, respectively), through TaqMan real-time PCR. Caco-2 and HCT-116 expressed different sets of miRNAs after treatment. Specifically, 21 and 22 miRNAs were differentially expressed in Caco-2 or HCT-116, respectively (t test, P < 0.01). By testing the expression of differentially expressed miRNAs in CRC patients, we found that miR-146b-3p and miR-486-5p are more abundant in K-ras–mutated samples with respect to wild-type ones (Wilcoxon test, P < 0.05). Sixty-seven percent of differentially expressed miRNAs were involved in cancer, including CRC, whereas 19 miRNA targets had been previously reported to be involved in the cetuximab pathway and CRC. We identified 25 transcription factors putatively controlling these miRNAs, 11 of which have already been reported to be involved in CRC. On the basis of these data, we suggest that the downregulation of let-7b and let-7e (targeting K-ras) and the upregulation of miR-17* (a CRC marker) could be considered as candidate molecular markers of cetuximab resistance. Global network functional analysis (based on miRNA targets) showed a significant overrepresentation of cancer-related biological processes and networks centered on critical nodes involved in epidermal growth factor receptor internalization and ubiquitin-mediated degradation. The identification of miRNAs, whose expression is linked to the efficacy of therapy, should allow the ability to predict the response of patients to treatment and possibly lead to a better understanding of the molecular mechanisms of drug response. Mol Cancer Ther; 9(12); 3396–409. © 2010 AACR.

Introduction

Colorectal cancer (CRC) is the third most common malignancy and cause of cancer-related deaths in Western societies (1). CRC tumors are characterized by multiple genetic alterations that are associated with specific clinical and pathologic changes, as described in the model proposed by Vogelstein et al. (2). Almost 85% of CRCs show high chromosomal instability with associated aneuploidy and loss of heterozygosity, whose degree is linked to clinical outcome (3). Furthermore, the CRC genome contains many genetic alterations, including activating mutations of proto-oncogenes (e.g., K-ras or B-raf) and inactivating mutations of tumor suppressor genes (e.g., APC and TP53; ref. 4). All these mutations are diagnostic and prognostic markers useful for CRC characterization (5). A common pharmacologic approach to CRC treatment is based on blocking the epidermal growth factor receptor (EGFR) signaling pathway by using monoclonal antibodies against EGFR (e.g., cetuximab, panitumumab). When EGFR is blocked, K-ras does not transduce proliferation signals and duplication of tumor cells is affected. However, K-ras mutations, casually occurring in CRC patients, may lead to a substitute in K-ras protein permanently turned on together with its downstream signaling. This may cause tumor

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-10-0137
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cells to proliferate without control. Our actual knowledge on CRC molecular features is the result of high-throughput expression arrays and genome-wide association studies that have generated novel markers and gene signatures (4, 5). Unfortunately, these are not sufficiently robust or validated to be clinically useful as predictive markers, except for the identification of K-ras mutations to predict the response of patients to cetuximab therapy (6). MicroRNAs (miRNA) are 21- to 24-nucleotide regulatory RNAs that negatively control the expression of about 30% of all protein-coding genes. They are involved in the regulation of almost every cellular process investigated so far (7). Accordingly, their mutations or altered expression correlate with various human diseases, including cancer (7). In the last few years, the global gene expression approach has been applied to identify CRC-specific miRNA signatures (8, 9), as well as in other solid cancers, with the intent to discover new diagnostic and prognostic markers (10). Many studies showed that miR-15b, miR-21, miR-181b, miR-191, and miR-200c may play a role in CRC development and progression and could be considered good markers for CRC prognosis (10, 11). Finally, a more recent study has shown that miR-17 and miR-92 are significantly elevated in the plasma of CRC patients and could be potential noninvasive markers for CRC diagnosis (12). Despite these advances, the relationship between therapeutic response and miRNAs expression in CRC still remains unknown. In this article, we report the results of TaqMan real-time PCR expression profile analysis on 667 miRNAs in sensitive and resistant human colon carcinoma cell lines before and after therapeutic treatment with cetuximab. Our aim was 2-fold: (i) to investigate miRNAs as markers of patients’ response to therapy; (ii) to analyze miRNA involvement in CRC drug response.

Materials and Methods

Cell lines

Caco-2 cells, with a wild-type K-ras genotype and cetuximab-responsive, and HCT-116 cell line, harboring a mutated K-ras and resistant to cetuximab, were from the Interlab Cell Line Collection (ICLC), an International Repository Authority within the Istituto Nazionale per la Ricerca sul Cancro (Genoa, Italy). The characterization and validation of both cell lines were performed by the cell bank. The cell lines were verified to be mycoplasma free by Hoechst staining and PCR (TIB Molbiol) and also by MycoTect (Gibco BRL). Species verification was performed by isoenzyme analysis (AuthentiKit TM System, Innovative Chemistry). Multiplex short tandem repeat profiling was performed to verify the identity and uniqueness of cell lines. After receiving the cell lines from ICLC, an aliquot was immediately frozen whereas the other was cultured up to the 10th passage thereafter to perform the experiments. Caco-2 cells were cultured in Eagle’s minimum essential medium (Cambrex Bio Science), supplemented with fetal bovine serum (FBS) 20%, 2 mmol/L of l-glutamine, and NEEA 1% (Gibco); HCT-116 cells were cultured in McCoy’s 5A medium (Gibco), supplemented with FBS 10% and 2 mmol/L of l-glutamine (Gibco).

Determination of chemosensitivity to cetuximab and panitumumab

The MTT assay was performed to determine the effects of cetuximab and panitumumab on cell viability. For each cell line, 5 × 10⁶ cells per well were seeded in 96-well plates in serum starvation conditions (1% FBS) and treated with 20 μg/mL of cetuximab (Erbilux; Merck KGaA) or 20 μg/mL of panitumumab (Vectibix; Amgen Europe B.V.). The MTT assay was performed at 3 time points (24, 48, and 72 hours posttreatment) after drug exposure of treated cells and their time-matched controls. For each cell line, a 96-well plate was harvested to assess the absorbance values at the starting point (t = 0). Absorbance values were read by using the Multiscan Ascent microplate reader (Thermo Fisher Scientific Inc.). All the experiments were performed in triplicates. These experiments were also performed in triplicates in 75-cm² flasks (1.5 × 10⁶ cells per flask) for molecular analysis.

Tissue samples

CRC samples, used in this study, were collected as part of standard clinical care activity. Formalin-fixed, paraffin-embedded (FFPE) samples from 25 patients were obtained from the Unit of Pathology, Azienda Polyclinico, University of Catania, and were analyzed in accordance with the policies of the institutional review board of the hospital. All the specimens were from surgical resecion and verified by histopathologic confirmation of neoplastic phenotype. For the isolation of DNA and total RNA, 20-μm tissue sections were used.

RNA isolation, reverse transcription, and miRNA profiling by real-time PCR

Total RNA from cell lines was extracted using TriZol reagent (Invitrogen), according to the manufacturer’s instructions. One microgram of total RNA was retrotranscribed using the TaqMan MicroRNA Reverse Transcription Kit and Megaplex Primer Pools (Applied Biosystems), according to the Megaplex Protocol. Six microoliters of each real-time product from both primer pools was mixed up with TaqMan Universal PCR Master Mix (Applied Biosystems) and loaded into the microfluidic cards TaqMan Human MicroRNA Array v2.0 A and B (Applied Biosystems). All reactions were performed in a 7900HT Fast Real Time PCR system (Applied Biosystem). All the experiments were performed in biological triplicates.

Expression data analysis

We applied 2 different methods for data normalization. First, we calculated the relative levels of miRNAs.
expression through the $2^{-\Delta CT}$ method. Data normalization was performed by using snRNA U6 and snRNA U48 as endogenous controls. The values of Ct greater than 35 were treated as 35, whereas underdetermined Ct values were treated as 40. We accepted as reliable only fold change values concordant in both endogenous controls. Expression data in Results section are shown as natural logarithm (ln) of relative quantity (RQ) of miRNAs, normalized with respect to snRNA U6 from a calibrator sample (the time-matched untreated controls). To identify differentially expressed miRNAs, we applied a paired t test (or t test between subject in the comparison between cell lines) among $\Delta$Ct by using the following parameters: assumption of equal variance; $P$ (overall threshold $P$ value) = 0.01; the $P$ value was based on permutation; the significance was determined by the adjusted Bonferroni correction (13). We considered as up- or downregulated those genes that have a natural logarithm of expression fold change of at least 1 or less, respectively. We excluded as differentially expressed miRNAs those miRNAs that have either undetermined Ct or Ct values of greater than 35 in both the sample and the calibrator. We also performed a second normalization with the global median normalization method. Similar to microarray analysis, for each sample, Ct values were normalized with respect to the median of Ct of the array (14). Data about the relative abundance of miRNAs in cell lines, before and after treatment, were displayed with MeV04 (http://www.tm4.org/mev.html).

**Detection of K-ras mutations and microRNA expression assays in FFPE CRC samples**

DNA and total RNA from FFPE CRC samples were isolated by using QIaAmp DNA kits (Qiagen) and Recover All Total Nucleic Acid Isolation Kit (Ambion), respectively, following the manufacturer’s protocol. The mutational status of K-ras in FFPE samples was assessed by using real-time PCR allelic discrimination. DNA from each sample was analyzed by TaqMan® MGB custom assays (Applied Biosystems) discriminating the 7 most common mutations in codons 12 and 13 of K-ras (15). We also assayed the K-ras genotype in both cell lines. The expression of miRNA from FFPE CRC samples was analyzed by TaqMan MicroRNA Assay (Applied Biosystems). Differential expression of miRNAs in patients with wild-type or mutated K-ras was evaluated by the Wilcoxon rank sum test ($P < 0.05$). All experiments were performed in triplicates.

**MicroRNA target prediction and gene ontology term analysis**

Predicted and experimentally validated targets of differentially expressed miRNAs were extracted from miREcords (http://mirecords.biolead.org). Predicted targets were considered reliable if identified at least by 4 to 6 prediction tools of a set of 11 established tools. To improve our target prediction, we also used anticorrelation data between miRNAs and mRNAs or miRNAs and proteins (where available refs. 16, 17; http://discover.ncbi.nlm.gov/cellminer/home.do). This additional filtering was also performed by using the Hoctar database for intronic microRNAs (http://hoctar.tigem.it). Standard Pearson correlation test was performed to verify the negative correlation between miRNAs and mRNAs by using $P < 0.05$. The Gene Ontology (GO) functional classification of miRNA targets was performed by using cytoscape plug-in BINGO (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html). The analysis of statistically significant GO differences between time points was performed by using Babelomics tool FatiGO (http://babelomics3.bioinfo.cipf.es).

**Computational genomic analysis**

The genomic position of human miRNA genes and their assignment to specific families were from CoGeMiR (http://cogemir.tigem.it). miRNA clusters include genes with maximum distance of 5,000 bp and were extracted from MirGen (http://www.diana.pcbi.upenn.edu/mirGen.html). To evaluate the presence of common regulatory mechanisms of miRNA clusters or families, we examined the relationship between genomic proximity of genes encoding miRNAs and their common expression by comparing their genomic positions and their expression values. Common potential transcription factors of differentially expressed miRNAs were from databases containing predicted, evolutionarily conserved transcription factors binding sites (TFBS) and validated transcription factors controlling miRNAs: TransMir (http://202.38.126.151/hmdd/mirna/tf/); PuTmIR (http://www.isical.ac.in/~bionfo_miru/TF-miRNA.php); and mirGen 2.0 (http://diana.cslab.ece.ntua.gr/mirgen/). The prediction of transcription factors was improved by verifying the correlation of expression (positive or negative) between the predicted couples miRNA/TF. The microarray data sets, used for this analysis, and statistical tests applied were the same as those described for target prediction.

**Network analysis**

The biological networks of miRNA target genes were built by retrieving the corresponding interactome data through cytoscape plug-in API2NET (http://bioinfow.dep.usal.es/apid/apid2net.html). They include validated differentially expressed miRNA targets, predicted targets from our anticorrelation analysis, and first neighbors of the targets. The analysis of network centrality was performed using the plug-in Network Analyzer (http://med.bioinf.mpi-inf.mpg.de/netanalyzer). Data on functional relationship between genes and drugs were extracted from the PharmGKB (http://www.pharmgkb.org/index.jsp) and Comparative Toxicogenomics Database (http://ctd.miblb.org/), whereas the information about cancer involvement was obtained from Database of Functional Censuses of Human Cancer Genes (http://bioinfo.hrbmu.edu.cn/fencens/Home.jsp), mi2Disease database, and literature (http://www.mi2disease.org).
Results

Differential sensitivity of colon cancer cells to cetuximab and panitumumab

To test the sensitivity of CRC cell lines to cetuximab and panitumumab, Caco-2 and HCT-116 viability after drug treatment was analyzed along a time course of 72 hours by using the MTT assay. As shown in Supplementary Data 1, cetuximab and panitumumab had an evident negative effect on Caco-2 cell line viability; for both EGFR inhibitors, the effect was time dependent. On the other hand, the MTT assay showed that both cetuximab and panitumumab have no appreciable effects on HCT-116 viability. We chose to analyze the effect of cetuximab on miRNA expression because its use is more common in the clinical setting and its differential effects on the 2 cell lines were more marked.

MicroRNA expression in CRC cell lines after cetuximab treatment

To identify differentially expressed miRNAs after cetuximab treatment in colon cancer cell lines with different drug sensitivity, we analyzed the expression profile of 667 miRNAs in Caco-2 and HCT-116 cells, 24 and 48 hours after drug treatment, by using TaqMan low-density array (TLDA) technology. To assess the reproducibility of TaqMan TLDA and show the robustness of the data, we computed the Pearson correlation (r; P < 0.01) among the ΔCt of biological replicates of treated and untreated cell lines. We found a high concordance between the 3 biological replicates for each sample (mean of r = 0.87 ± 0.07 Supplementary Data 2). The values of r were generated by using the ΔCt of all miRNAs analyzed through fluidic cards. By removing Ct values of greater than 35 from this analysis, the mean value was r > 0.95 ± 0.03. The results obtained using 2 different normalization methods are comparable with each other; however, we selected the 2ΔΔCt method for further analysis, as it was shown to be more stringent (Supplementary Data 4). Figure 1 shows the patterns of expression of 667 human miRNAs in Caco-2 and HCT-116 after 24 and 48 hours of 20 µmol/L of cetuximab treatment, and their time-matched controls. At 24 hours posttreatment, there were 12 differentially expressed miRNAs (6 downregulated and 6 upregulated) in Caco-2 cells (Table 1), whereas in HCT-116 cells, we found 16 differentially expressed miRNAs (11 downregulated and 5 upregulated) 24 hours after cetuximab treatment (Table 1). The sets of differentially expressed miRNAs at 24 hours posttreatment were different in the two cell lines, except for miR-330-5p and miR-610, which were upregulated and downregulated, respectively, in both cell lines. At 48 hours posttreatment, the number of differentially expressed miRNAs in Caco-2 decreased, as we observed 10 miRNAs with altered expression (8 downregulated and 2 upregulated; Table 1), whereas we found 6 differentially expressed miRNAs (5 downregulated and 1 upregulated) at 48 hours posttreatment in HCT-116 (Table 1). As previously observed at 24 hours posttreatment, the sets of differentially expressed miRNAs in Caco-2 and HCT-116 at 48 hours posttreatment were not overlapping, confirming that the 2 cell lines respond differently to cetuximab treatment, as already shown by MTT experiments. It is interesting to note that miR-610 was downregulated in both cell lines at 24 and 48 hours posttreatment (with exclusion of HCT-116 at 48 hours posttreatment); miR-219-1-3p was downregulated in Caco-2 and HCT-116 at 24 and 48 hours posttreatment, respectively; miR-122, overexpressed in Caco-2 at 24 hours posttreatment, showed decreased expression in the same cell line at 48 hours posttreatment; and miR-17* was upregulated in HCT-116 at 24 hours posttreatment, but downregulated in Caco-2 at 48 hours posttreatment. The altered or antithetic expression of these miRNAs at 24 and 48 hours posttreatment in the 2 cell lines could suggest their important role within the signaling cascade triggered by cetuximab. On average, about 35% of the differentially expressed miRNA genes in Caco-2 and HCT-116 at 24 hours posttreatment showed the same expression trend at 48 hours posttreatment (Fig. 2). This shows that part of miRNA regulation began at 24 hours posttreatment and continued until 48 hours posttreatment. The significance level of some of these data was below our stringent threshold of acceptability, and for this reason they were not reported in Table 1. By comparing the RQ of miRNAs at steady state in both cell lines, we found that about 7% of all miRNAs analyzed had different expression levels in the 2 cell lines. This suggests that the corresponding molecular networks at steady state may be differentially prone to respond to EGFR inhibitors (Supplementary Data 3). Furthermore, about 8% (let-7b, miR-146a, miR-330-3p, miR-486-5p) of differentially expressed miRNAs in treated Caco-2 and HCT-116 also seemed to be differentially expressed in the 2 cell lines at steady state (Table 1).

Correlation between mutational status of K-ras and miRNA expression in FFPE CRC samples

In our cohort of CRC patients, we found 14 patients with wild-type K-ras and 11 with mutated K-ras (Supplementary Data 4). From the lists of differentially expressed miRNAs and unchanged miRNAs, we chose a set of 10 miRNAs to be tested in these patients: miR-17a, miR-19a*, miR-99b, miR-146b-3p, miR-194, miR-219-1-3p, miR-330-5p, miR-486-5p, miR-497, and miR-708. Our data indicated that the expression of miR-17a, miR-19a*, miR-99b, miR-194, miR-219-1-3p, miR-330-5p, miR-497, and miR-708 had no significant difference between patients with wild-type or mutated K-ras (Wilcoxon rank sum test: P = 0.42 for miR-17a; P = 0.27 for miR-19a*; P = 0.11 for miR-99b; P = 0.18 for miR-194; P = 0.34 for miR-219-1-3p; P = 0.36 for miR-330-5p; P = 0.5 for miR-497; and P = 0.34 for miR-708). On the contrary, miR-146b-3p and miR-486-5p had expression levels significantly elevated in CRC samples with mutated K-ras when compared with wild-type K-ras CRC samples (Wilcoxon rank sum test: P < 0.05 for miR-146b-3p; P < 0.01 for miR-486-5p).
Figure 1. miRNA transcriptome in treated and control CRC cell lines. Expression matrix of 667 miRNAs after 24 and 48 hours in cetuximab-treated Caco-2 and HCT-116 cells and relative controls. Data are shown as average $\Delta$Ct normalized using snRNA U6 as endogenous control. Rows, miRNA; columns, samples. $\Delta$Ct are shown according to the shading gray bar shown below the matrix.
miR-18b, miR-19b-2, miR-20b, miR-92-2, miR-106a, and miR-18a* were upregulated in Caco-2 after cetuximab treatment. For example, miR-194/miR-215 cluster on chromosome 1 and miR-372/miR-373 cluster on chromosome 10 were expressed at lower levels in HCT-116 during cetuximab administration. For example, miR-99b/let-7e and let-7a/let-7b were downregulated in HCT-116 during cetuximab administration. This could be due to their mapping to the same chromatin domain or to their sharing the same promoter region. On the contrary, the expression of miRNAs from the largest clusters was different (Supplementary Data 5a). We performed the same analysis on miRNA families located in different genomic loci. We identified 22 families (comprising 39 miRNAs), which showed different expression patterns between cell lines at steady state or after treatment (Supplementary Data 5b). On the contrary, the families of miR-99, miR-152, and miR-290 showed similar expression at steady state. Interestingly, let-7b and let-7e (let-7 family) and miR-148a and miR-148b (miR-148 family) maintained the same trend of expression after cetuximab treatment, even if they were not included in the differentially expressed class because of our stringent test: $P < 0.03$ and $P < 0.006$, respectively; Fig. 3). miR-486-5p was also more expressed in HCT-116 (with K-ras mutated) than in Caco-2 (with wild-type K-ras). Both these miRNAs were upregulated in Caco-2 after cetuximab treatment.

### Regulation of miRNA families and genomic clusters

To identify the hypothetical presence of miRNA cluster regulation, we analyzed the expression of neighboring miRNAs (separated by $<5$ kb). We found that miRNAs localized within the same narrow genomic region had similar trends of expression in the comparison between cell lines at steady state; miRNAs localized near differentially expressed miRNAs had a similar expression trend after treatment, even if they were not included in the differentially expressed class because of our stringent threshold. For example, miR-194/miR-215 cluster on chromosome 1 and miR-372/miR-373 cluster on chromosome 19 were expressed at higher levels in Caco-2 with respect to HCT-116; on the contrary, the cluster including miR-18b, miR-19b-2, miR-20b, and miR-363 on chromosome X was expressed at lower levels in Caco-2 with respect to HCT-116 (Additional File 5a). In most cases, miRNAs in close genomic proximity ($0.1–1$ kb) maintained the same trend of expression after cetuximab administration. For example, miR-99b/let-7e and let-7a/let-7b were downregulated in HCT-116 during treatment. This could be due to their mapping to the same genomic region or their sharing of common regulatory signals.

### Identification of putative conserved TFBS for coregulated miRNAs

To find common regulatory mechanisms controlling miRNA expression after treatment, we searched for validated or predicted TFBSs in genomic regions upstream of miRNA genes and evaluated the expression correlation between transcription factors and miRNAs. We identified 203 transcription factors, among which we selected 25 for their high number of occurrences and their involvement in CRC pathogenesis.
and in pharmacologic response (Supplementary Data 6). We filtered and validated the data on TFBSs through the analysis of expression correlation among transcription factors and miRNAs. This allowed us to identify HNF4A, RUNX1, and TCF3 as transcription factors controlling the highest number of differentially expressed miRNAs (≥15) in both cell lines after treatment. We also found that some transcription factors, related to organ development or involved in different cancers (i.e., FOXD3, GATA1, HNF1A, MAF, NKX2-5, PAX4, and POU2F1), had a high occurrence of TFBS and were correlated with the expression of differentially expressed miRNAs. On the basis of these findings, we hypothesize their potential involvement also in CRC response to cetuximab. As expected, many differentially expressed miRNAs are controlled by CRC-related transcription factors: MYC, amplified and overexpressed in CRC; TCF3, controlled by the WNT pathway; YY1 and ZEB1 overexpressed in CRC; HAND1 and PAX6, frequently hypermethylated in CRC; JUN, activated by K-ras cascade; MYB, activated in CRC; and TP53, deleted or mutated in CRC (Supplementary Data 6). Interestingly, we found that some of the genes, controlled by the K-ras pathway and involved in cetuximab response, potentially control the expression of differentially expressed miRNAs. For instance, ELK1 putatively regulates miR-193b* and miR-523 and is positively correlated to their expression; STAT1, activated by EGF signaling, could be responsible for miR-146b-3p abundance in K-ras–mutated FFPE samples; in K-ras wild-type cell line, where miR-145 (targeting STAT1) is downregulated, STAT1 potentially activates miR-146b-3p after 48 hours of cetuximab exposure. By mining TransMir, we found that MYC represses expression of let-7b and let-7e (their pre-miR also had anti-correlated expression with MYC) and could be

Figure 2. Differentially expressed miRNAs after cetuximab treatment. Expression trend of differentially expressed miRNAs in Caco-2 and HCT-116 after 24 and 48 hours of cetuximab administration (paired t-test, P < 0.01). Data are shown as average natural logarithm (ln) of relative quantity of miRNAs in treated cells with respect to the time-matched untreated control. A, differentially expressed miRNAs in Caco-2 after 24 hours; B, differentially expressed miRNAs in HCT-116 after 24 hours; C, differentially expressed miRNAs in Caco-2 after 48 hours; and D, differentially expressed miRNAs in HCT-116 after 48 hours.
responsible for downregulation of these miRNAs after 24 hours of treatment. Finally, miR-17* expression during cetuximab treatment in Caco-2 and HCT-116 could be due to the regulation of miR-17-92 cluster by MYC and NK-like family of homeobox genes (NKX2-5, TLX1, and TLX3).

**MicroRNA targets**

To evaluate the biological relevance of differentially expressed miRNAs identified in our analysis, we searched for their dysregulation in human diseases (see the Materials and Methods section). We found that 67% of differentially expressed miRNAs are involved in cancer and 20% of them are related to CRC. Moreover, 50% of miRNAs that were differentially expressed between two cell lines at steady state were involved in CRC. We identified 513 differentially expressed miRNAs targets (226 experimentally validated and 287 predicted; Supplementary Data 7). All predicted targets, selected for further analysis, had a negative Pearson coefficient in the correlation analysis with miRNA expression ($P < 0.05$). We found that about 10% of these targets were cancer-related genes and among them about 30% were involved in CRC. Moreover, we found that about 36% of targets had been previously reported to be involved in CRC response to drugs and 11 targets had been previously reported to be involved in the cetuximab pathway (Table 2).

**MicroRNA target-centered network and gene ontologies**

We built the networks of all differentially expressed miRNAs after treatment, in both cell lines for each time point, by retrieving protein–protein and DNA–protein interactions of their targets and first neighbors. The networks were made of 2,594, 712, 2,244, and 839 nodes in Caco-2 at 24 and 48 hours posttreatment and in HCT-116 at 24 and 48 hours posttreatment, respectively. Interestingly, we found that AKT1, GRB2, MYC, NFKB2, PLCG1, RAF1, and SP1, which are all related to the cetuximab pathway, are nodes shared by all the networks and, in most of the cases, are also the network hubs (>90 interactions); moreover, 6.7%, 6.7%, 7.2%, and 8.7% of genes in Caco-2 at 24 hours, Caco-2 at 48 hours, HCT-116 at 24 hours, and HCT-116 at 48 hours posttreatment, respectively, are involved in several types of cancer, including CRC; 23.2%, 20.8%, 23.5%, and 26.5% of these genes are involved in CRC response to drug treatment. The analysis of network centrality showed that EEF1A1, GRB2, and UBC (that are interactors of miRNA targets) are amongst the proteins with the highest degree in all networks. It is interesting to note that GRB2 (332, 97, 254, and 123 interactions in Caco-2 at 24 and 48 hours posttreatment and HCT-116 at 24 and 48 hours posttreatment, respectively) was previously reported to be involved in cetuximab response and, more
Table 2. miRNA target genes previously reported to be involved in cetuximab pathway, CRC, and most frequently involved in CRC response to drugs

<table>
<thead>
<tr>
<th>Target genes Differentially expressed miRNAs validated or predicted to target mRNAs</th>
<th>Caco-2 expression</th>
<th>HCT-116 expression</th>
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<tr>
<td><strong>miRNA targets previously reported to be involved in the cetuximab pathway</strong></td>
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<tr>
<td>C18orf37 hsa-miR-124</td>
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<td>CCND1 hsa-let-7b</td>
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<td>PTPN12 hsa-miR-486-5p</td>
<td>Up, 24 h PT</td>
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<tr>
<td>PTPN12 hsa-miR-124</td>
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<tr>
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<tr>
<td>CCND1 hsa-let-7b</td>
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<tr>
<td>MAP2K4 hsa-miR-576-3p</td>
<td>Up, 48 h PT</td>
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<td>N-ras hsa-let-7b</td>
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<tr>
<td>PRKCA hsa-miR-330-5p</td>
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<tr>
<td><strong>miRNA targets previously reported to be involved in CRC response to drugs</strong></td>
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<td>RELA hsa-miR-124</td>
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<tr>
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<td>CTGF hsa-miR-124</td>
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(Continued on the following page)
generally, in CRC response to drugs (18). EEF1A1 is considered to be a regulator of cell growth and cytoskeletal network; its expression is elevated in tumors (including CRC) and it was reported to be involved in CRC response to drugs (19–21). UBC is a component of the ubiquitin proteasome system that plays a key role in the regulation of colorectal epithelial cells proliferation, and is thus implicated in colorectal carcinogenesis (22). We determined the statistically overrepresented GOs in the 4 networks, focusing on the comparisons between the 2 cell lines on a given time point. This analysis highlighted a high occurrence of common processes such as the regulation of transcription, regulation of cell proliferation, cell motility and angiogenesis, regulation of mitogen-activated protein kinase (MAPK) activity, apoptosis at 24 hours posttreatment, whereas at 48 hours posttreatment, the 2 cell lines share only general processes as transcription, regulation of cell proliferation, and apoptosis (hypergeometric test, Benjamini adjusted \( P < 0.00005 \)).

Interestingly, we observed that at 24 hours posttreatment, GOs in Caco-2 are enriched in proteins related to the activation of apoptotic process, differentiation, and negative regulation of cell growth and migration, regulation of MAPK activity, Wnt and Notch pathways and vascular endothelial growth factor (VEGF) activity, apoptosis at 24 hours posttreatment, whereas at 48 hours posttreatment, the 2 cell lines share only general processes as transcription, regulation of cell proliferation, and apoptosis (hypergeometric test, Benjamini adjusted \( P < 0.05 \), Fig. 4). The processes correlated to immunity are extensively represented in our GO analysis. With respect to HCT-116, Caco-2 are enriched in proteins involved in the activation of the molecular mediators of inflammation both at 24 and 48 hours posttreatment (Fig. 4). Moreover, in both Caco-2 at 24 and 48 hours posttreatment, we found that several GOs related to NF-κB activity are absent in HCT-116 (Fig. 4). We identified the statistically significant differences in GO terms between 24 and 48 hours posttreatment. In Caco-2 cells at 24 hours posttreatment, we found a high occurrence of biological processes and molecular functions related to signal transduction, transmembrane receptor activity, and G-protein–coupled receptor protein signaling pathway in comparison with 48 hours posttreatment (Fisher exact test, adjusted \( P < 0.05 \)).

**Discussion**

The molecular bases of response to cancer therapeutics are complex because they involve multiple processes and corresponding cellular pathways and networks (23, 24). The new generation of targeted therapies has been conceived to affect specific molecular processes that control tumor growth, survival, and apoptosis (25). Several anti-tumor agents, interfering with the EGFR pathway, have been developed (25). Among them, cetuximab is used for CRC treatment either as a single agent or in combination with chemotherapy (26). The mechanisms through which cetuximab performs its anticancer activity are numerous...
Figure 4. Matrix of the differences of Gene Ontology among networks based on differentially expressed miRNA target. Networks of differentially expressed miRNA targets include validated targets, predicted targets having anticorrelated expression with their miRNAs and targets’ first neighbors. GO functional classification and $P$ value of overrepresented GO calculation was performed by using Cytoscape plug-in BINGO (hypergeometric test, adjusted $P < 0.05$). Rows, GO ID; columns, samples. Scale bar on the top of the figure represents the –log of $P$ value. Black, gene ontologies absent in network; grey shade, gene ontologies present in network, according to the scale bar. Abbreviation: TGF, transforming growth factor.
and not completely understood. They include inhibition of cell-cycle progression, angiogenesis, invasion and metastatization, activation of apoptosis, and synergic cytotoxicity with chemotherapy and radiotherapy (27). Genome-wide expression profiling of mRNAs, triggered by drugs, has been used to understand the regulatory circuits. It has been noticed that the levels of mRNAs and the encoded proteins are often not overlapping. This could have a number of causes, such as posttranscriptional regulation (13). In this context, miRNAs could have a critical role in controlling the complex signaling activated by cues internal and external to the organism, including antitumoral therapeutic strategies (28). miRNA expression is strongly implicated in CRC tumorigenesis and progression (29).

Accordingly, we hypothesized that they could incisively affect the response to cetuximab. Indeed, the expression data reported in this article show that cetuximab treatment induced miRNA transcriptome changes in drug-sensitive and drug-resistant CRC cells (Fig. 1, Table 1). These expression variations involved single-copy miRNAs as well as miRNAs from gene clusters or gene families (e.g., let-7 and miR-148 families; Supplementary Data 5a and 5b). Interestingly, the set of differentially expressed miRNAs in the 2 cell lines was almost entirely not overlapping, with exclusion of miR-219-1-3p, miR-330-5p, and miR-610 (Table 1). These data would indicate a different miRNA transcriptome organization in the 2 model systems because of the specific and expected genome individuality that they mirror. These differences could affect the relative molecular networks and trigger different responses to EGFR inhibitors; for instance, the constitutive activation of K-ras in HCT-116 could lead to a different regulation of miRNA signaling with respect to cells with wild-type K-ras. Unsurprisingly, let-7b and let-7e are downregulated in HCT-116 after cetuximab treatment. The members of let-7 family are known to target K-ras and, recently, their involvement in cetuximab response has been reported (30). After cetuximab treatment, in resistant CRC cell lines, the signaling downstream of K-ras remains activated. The downregulation of its negative regulators (let-7) could be a mechanism positively selected in these cells to contribute to cetuximab resistance. The different miRNA transcriptomes in the 2 cell lines could be the outcome of transcription factors involved in the response to cetuximab or in related pathways. On the basis of our data, we found 25 putative miRNA-controlling transcription factors for both cell models (e.g., HNF4A, RUNX1, TCF3), 17 of which had been previously reported to be involved in CRC (e.g., MYC, TCF3; refs. 31, 32; Supplementary Data 6). Some of these transcription factors could specifically control the expression of differentially expressed miRNAs, determining the variation of miRNA transcriptome after cetuximab treatment (33). We identified a reliable set of miRNA targets of differentially expressed miRNAs. These may provide further insight into the molecular mechanisms and biological pathways responsible for sensitivity or resistance to therapy (Supplementary Data 7). Intriguingly, all of these miRNAs had trends of expression opposite to their mRNA targets (negative Pearson coefficient). This suggests that these miRNA–mRNA couples may correspond to biological relevant regulatory circuits in our models (34). Indeed, the miRNAs identified in this study are targeted by several differentially expressed miRNAs having well-established CRC associations (i.e., let-7, miR-17*, miR-18a*, miR-107, miR-133b, miR-223) and some of them are known to be involved in the cetuximab pathway (let-7b, let-7e; Table 2). It is notable that miR-17* (previously known as miR-17-3p), a CRC marker found to be abundant in biopsies and plasma of patients and positively regulated by MYC, is upregulated in the resistant cell line and downregulated in the sensitive one after cetuximab treatment. This antithetic modulation of miR-17* could represent one of the discriminating molecular mechanisms of resistance or sensitivity. The higher levels of miR-146b-3p and miR-486-5p in K-ras-mutated samples, with respect to wild-type ones, could be the indirect result of constitutional activation of K-ras signaling (Fig. 3). Almost 80% of the miR-486-5p targets are negative regulators of cell proliferation or involved in apoptosis (e.g., ARHGAP5 negatively regulates RHO GTPases; DOCK3 is an inhibitor of Wnt/beta-catenin signaling; TOB1 is an antiproliferative protein-controlling cell growth; ST5 is a negative regulator of MAPK1/ERK2 kinase; refs 35–38). Moreover, among the targets of miR-486-5p, there is PIK3R1 that is known to be upregulated in responsive patients after cetuximab treatment (39). miR-146b-3p has been reported to be an unfavorable cancer prognostic marker for patients with squamous cell lung cancer or papillary thyroid carcinoma (40–42). Interestingly, its upregulation is statistically associated with BRAF mutation in papillary thyroid carcinoma. BRAF is a downstream effector of K-ras. Its mutations are reported in CRC patients and are mutually exclusive with respect to those of K-ras (42). Moreover, indirect evidence in breast cancer would suggest that miR-146a and 146b target EGFR (43). Taken together, these data suggest that the upregulation of miR-146b-3p and miR-486-5p in CRC patients with mutated K-ras would be a downstream molecular effect of hyperproliferative stimuli triggered by K-ras mutations or in other nodes from the same pathway. Accordingly, profiling of these miRNAs in CRC patients could complement K-ras–based diagnosis to predict sensitivity to cetuximab. Unsurprisingly, the 4 networks of differentially expressed miRNA targets and their first interactors are centered on the same hubs, EEF1A1, GRB2, and UBC. Although the role of EEF1A1 in CRC remains unsatisfactorily characterized, the involvement of GRB2 and UBC in the regulation of EGFR pathway has been proved (22, 44). In particular, GRB2 is a critical link between cell surface growth factor receptors and the RAS signaling pathway, participating in EGFR internalization and its subsequent ubiquitylation (44). Moreover, the ubiquitin complex regulates other key molecules in CRC carcinogenesis and components of the K-ras pathways. This would mean that the regulatory circuits spreading from differentially
expressed miRNAs focus on key molecules, playing a central role in proper signaling transmission from EGFR and in its internalization and turnover. These findings are well inserted in the scenario of the downstream effects of cetuximab. Indeed, it has been shown that cetuximab also induces EGFR internalization and ubiquitin-mediated degradation. To determine the main biological and molecular functions affected by differentially expressed miRNAs, we analyzed the statistically overrepresented GO terms within the networks. We observed that differentially expressed miRNAs in Caco-2 both at 24 and 48 hours posttreatment preferentially target genes involved in the regulation of Notch and Wnt pathways, whereas the set of differentially expressed miRNAs in HCT-116 may control the signaling pathways of EGFR at 48 hours posttreatment (Fig. 4). In intestinal crypts, the canonical Wnt pathway inhibits the β-catenin destruction complex (45). The processes related to immunity are extensively represented in our GO analysis. We noticed that Caco-2 cells, with respect to HCT-116, are enriched in proteins involved in the activation of the molecular mediators of inflammation (Fig. 4). It has been reported that cetuximab treatment of CRC patients increases the expression of proinflammatory proteins and that host response to therapy could be a potential prognostic factor in CRC (39).

Moreover, in Caco-2 at 24 and 48 hours posttreatment, we found several GOs related to NF-κB activity, which are absent in HCT-116 (Fig. 4). Expression of this transcription factor, also activated by the EGFR downstream signaling pathway, has been associated with prognosis and resistance to antitumoral treatments in CRC and other solid tumors (46). These data stress as different responses to cetuximab are related to different sets of differentially expressed miRNAs and, accordingly, to different molecular signaling. Finally, we identified statistically significant differences in GO terms between 24 and 48 hours posttreatment in our model cell lines. Caco-2 showed at 24 hours posttreatment a significant occurrence of biological processes and molecular functions, related to signal transduction, transmembrane receptor activity, and G-protein-coupled receptor protein signaling pathway with respect to 48 hours posttreatment. This indicates that the regulation of the molecular cascade, controlling the EGFR-mediated pathway, represents an early effect of cetuximab administration in the sensitive cell line.

Conclusions and Perspectives

The miRNAs identified through our experiments possess the following features: The expression of some was altered in both CRC cell lines after cetuximab treatment (e.g., upregulation of miR-330-5p and downregulation of miR-610); the expression of others was specifically regulated in one cell line and clearly related to the cetuximab pathway (e.g., downregulation of let-7b and let-7e and upregulation of miR-17* in HCT-116); finally and most interesting, others were found to be more abundant in patients with mutated K-ras before treatment (i.e., miR-146b-3p and miR-486-5p). To date, the possibility of miRNAs involvement in patients’ response to cetuximab remained unexplored. Our data provide for the first time a systemic view of the participation of miRNAs in the molecular cascade triggered by cetuximab. They also suggest their exploitation as predictive markers of drug response. This could pave the way to larger epidemiologic studies and clinical applications on CRC specimens for pharmacoresistance screenings in patients, searching for an association of specific miRNA profiles to responsiveness classes for cetuximab. The identification of these miRNAs should eventually allow to foresee the response of patients to treatment and possibly lead to a better understanding of the molecular mechanisms of drug response. Finally, these studies could also identify chemoresistance-related miRNAs as pharmacologic targets for combining therapy with antagonists (47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We gratefully acknowledge the kind gift of Caco-2 and HCT-116 by Prof. Massimo Romani (IST, Genoa, Italy). Drs. L. Duro, M.R. Guglielmino, A. Majorana, R. Angelica, L. StTELlo, L. Salitio, and M. Maugeri are PhD students of the Dottorato di Ricerca in Biologia, Genetica Umana, Bioinformatica, Basi Moleculari e Cellulari del Fenotipo (Director: Prof. M. Purrello). The technical collaboration of Mrs. M. Cocimano, Mr. L. MessiNA, Mr. F. Mondio, and Mr. A. Vasta is acknowledged.

Grant Support:

This work was supported by Ministero dell’Università e della Ricerca Scientifica e Tecnologica by funds to Prof M. Purrello (FIRB 2007: Dalla Proteonomica alla Biologia Cellulare; FAR 2007: Generation of a technological platform to study the effects of anti-neoplastic drugs and to investigate their potential efficacy as neuroprotective agents; PRA 2007: Caratterizzazione delle Orme del Macchinario Apoptotico e dell’Apparato di Trascrizione: ruolo biologico dei microRNA e loro coinvolgimento in Patologia; Progetti Dipartimentali della Facoltà di Medicina e Chirurgia: BioMedicina Molleolare dei Sistemi Complessi: Analisi molecolare delle Orme nel Carcinoma del Colon e del Retto ed Applicazioni Cliniche) and PRA 2007 to Prof. F. Basile, Prof. A. Cappellani, Prof. M. Di Vita, and Prof. S. Lanuzafame.

Received 02/15/2010; revised 09/13/2010; accepted 09/17/2010; published 12/14/2010.


Molecular Cancer Therapeutics

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