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**Panitumumab and cetuximab affect differently miRNA expression in colorectal cancer cells**

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## 1 Structured abstract

### 2 Background and aim

3 Resistance to anti-EGFR monoclonal antibodies (mAb) in metastatic colorectal cancer  
4 is frequent and prognostic biomarkers are lacking. Micro-RNAs (miR) are good  
5 candidates in this context. We aimed to characterize cetuximab and panitumumab  
6 exposure influence on miR expression in colorectal cancer cells to identify those  
7 regulating the EGFR pathway and implicating in resistance to treatment. Finally we  
8 aimed to identify miR expression in serum of patients with advanced colorectal cancer  
9 treated with cetuximab or panitumumab.

### 10 Results

11 Cetuximab and panitumumab exposure induced significant expression variations of  
12 17 miR out of a miRnome panel of 752. 6 of those miR interacted with at least one  
13 downstream element of the EGFR pathway.

### 14 Conclusion

15 After the bioinformatics two-phase process, 5 miR rarely described before could be  
16 potential actors of anti-EGFR mAb resistance: miR-95-3p, miR-139-5p, miR-145-5p,  
17 miR-429 and miR-1247-5p. *In vivo*, we detected the expression of miR-139-5p and miR-  
18 145-5p in serum of patients with metastatic colorectal cancer.

19  
20 **Keywords:** microRNA, metastatic colorectal cancer, monoclonal antibodies,  
21 Epidermal Growth Factor Receptor, biomarkers, cell lines, serum

## 31 Introduction

32  
33 Colorectal cancer (CRC) is the third most common diagnosed cancer and the second  
34 cause of cancer-related death worldwide. It is a major public health concern. In more  
35 than half cases, it is complicated with distant metastasis which is a turning point in its  
36 evolution. Even though therapeutic progress has been made during the last two  
37 decades, CRC metastatic stage (mCRC) is of a bad prognosis. The therapeutic arsenal  
38 of mCRC combines local treatments with systemic ones based on chemotherapy and  
39 targeted therapies. The latter have taken a large place in mCRC management.  
40 Monoclonal antibodies (mAb) targeting the vascular epidermal growth factor (VEGF)  
41 or the epidermal growth factor receptor (EGFR) are now used as first line treatments.  
42 Their efficacy is particularly influenced by **tumor mutational** characteristics.

43  
44 CRC genesis is linked with the accumulation of gene expression deregulations [1]. The  
45 two main pathogenic paths are chromosomal instability and microsatellite instability.  
46 Progression and systemic dissemination of cancer cells are determined by the  
47 deregulation of intra-cellular signaling pathways and extra-cellular interactions. EGFR  
48 pathway plays a major role in CRC process. Epidermal growth factor's binding to its  
49 receptor activates the MAPK/ERK (mitogen-activated protein kinase/extracellular  
50 signal-regulated kinase), PI3K/Akt (phosphoinositidine 3-kinase/protein kinase B)  
51 and JAK/STAT (Janus kinase/signal transducers and actors of transcription) intra-  
52 cellular pathways. After endocytosis, EGFR can also act as transcriptional regulator  
53 for cell cycle influencing genes [2]. These activations boost proliferation, migration,  
54 cell division, tumoral angiogenesis and epithelial-mesenchymal transition [3].  
55 Deregulated EGFR activation gives colorectal cells a pro-tumoral phenotype. This  
56 receptor is therefore a relevant therapeutic target. Two mAb targeting the EGFR are  
57 widely used in mCRC treatment. Cetuximab is a chimeric IgG1 antagonistic mAb  
58 which competitively targets EGFR extra-cellular element. Its binding induces the  
59 internalization and degradation of the receptor. It also limits other activating ligands  
60 fixation by steric bulking [4]. Cetuximab can also recruit cytotoxic effective immune  
61 cells by its Fc portion [5]. Panitumumab is a human IgG2 antagonistic mAb which has  
62 a mechanism of action similar to that of cetuximab except its limited antibody-

1  
2  
3 63 dependent cellular cytotoxicity due to its IgG2 nature [6]. It was demonstrated that in  
4 64 clinical practice, these two anti-EGFR mAbs have a comparable therapeutic efficacy  
5 65 in mCRC [7]. They can both be used as first line treatments if associated with a  
6 66 cytotoxic chemotherapy regimen, as well as in maintenance of mCRC. However, put  
7 67 aside their established overall survival increase, resistance to cetuximab and  
8 68 panitumumab is frequent in mCRC [8]. Biomarkers predicting anti-EGFR mAb efficacy  
9 69 in order to improve patients' selection for treatment, is a challenge which has justified  
10 70 numerous studies. To identify these biomarkers, understanding the mechanisms of  
11 71 resistance to these mAbs is essential. It appears that resistance to cetuximab and  
12 72 panitumumab is due to quantitative (over expression or amplification) and/or  
13 73 qualitative (mutations) anomalies in EGFR or to the independent or constitutive  
14 74 activation of intra-cellular downstream effectors (RAS, RAF, PI3K, PTEN). The only  
15 75 factors of resistance to cetuximab and panitumumab clearly demonstrated in clinical  
16 76 practice are the activating mutations of *KRAS* and *NRAS* genes [9]. They are at the  
17 77 origin of a RAS protein which is activated independently of EGFR status. Hence,  
18 78 blocking the EGFR by mAbs does not influence the pro-tumoral impact of the  
19 79 independently activated RAS pathway. *KRAS* mutations occurs in 40% of patients  
20 80 with mCRC [10]. However, of the wild-*RAS* patients who are treated by anti-EGFR  
21 81 mAbs, only 40% have a positive survival outcome [8]. It is therefore essential to  
22 82 identify new predictive biomarkers of anti-EGFR MAb efficacy in order to optimize  
23 83 mCRC treatment strategy.

24 84  
25 85 MicroRNAs (miRNA or miR) are implicated in gene post-transcriptional regulation.  
26 86 They are short (8-22 nucleotides) single-stranded non-coding RNAs. They hybrid with  
27 87 target mRNA by complementarity of 6-8 nucleotides in the 3' untranslated transcribed  
28 88 region. When merged, the mRNA's transcription is repressed. A miR can regulate the  
29 89 expression of hundreds of genes and at the same time, one gene can be regulated by  
30 90 many miR [11]. More than 2500 mature miR have been discovered, targeting more than  
31 91 half of the entire human genome [11]. The implication of miR in human pathology has  
32 92 been particularly studied during the last decade. Their biogenesis in human eukaryote  
33 93 cells is complex and involves numerous elements which coordination can be damaged

1  
2  
3 94 causing deregulated miR production. Deregulated miR have an impact on diverse  
4  
5 95 human diseases and especially on cancers. Mechanisms of miR deregulations in cancer  
6  
7 96 cells are diverse [12]. Genetic alterations, preliminary transcriptional promoters'  
8  
9 97 deregulations, epigenetic alterations and dysfunction in the enzymatic machinery of  
10  
11 98 miR biogenesis, quantitatively and/or qualitatively modify miR influence [13]. As  
12  
13 99 such, some miR are deregulated favoring pro-tumoral processes (onco-miR): pro-  
14  
15 100 proliferation, anti-apoptotic, angiogenesis, epithelial-mesenchymal transition. Some of  
16  
17 101 these onco-miR influence cardinal colorectal cancer pathways such as the EGFR-  
18  
19 102 pathway, WNT/ $\beta$ catenin pathway, TGF- $\beta$ /Smad pathway or the MET pathways [14].  
20  
21 103

22 104 Some miR were shown to interact with the EGFR pathway in CRC but only few have  
23  
24 105 been associated with the efficacy of anti-EGFR mAb in clinical practice [15]. All of  
25  
26 106 those studied have been screened on primitive colorectal tumor tissue of patients  
27  
28 107 treated by anti-EGFR mAb after several chemotherapy regimens. It seems that  
29  
30 108 differential tumor expressions of miR-31-5p/3p, miR-592, miR-140-5p, miR-1224-5p,  
31  
32 109 miR-181a, miR-302, miR-100, miR-125b and miR-7 are associated with survival and/or  
33  
34 110 disease progression after cetuximab or panitumumab treatment [16-22]. However, the  
35  
36 111 heterogeneity of these findings and the intricated elements influencing miR  
37  
38 112 expression, makes it complicated to comprehend the underlying mechanisms of miR  
39  
40 113 impact on anti-EGFR mAb efficacy in mCRC. Thanks to nowadays bio-informatic tools  
41  
42 114 enriched by the growing interest of miR influence on cancer processes, this study aims  
43  
44 115 at understanding the influence of cetuximab and panitumumab exposure on miR  
45  
46 116 expression (MiRnome profiling) in colorectal cancer cells in order to identify those  
47  
48 117 implicated in the EGFR pathway and resistance to anti-EGFR mAbs ; as well as to  
49  
50 118 describe the possible differences in miR expression between these two anti-EGFR  
51  
52 119 mAbs. Finally we aimed to validate the expression of these selected miRs in serum of  
53  
54 120 patients with mCRC.  
55  
56 121  
57  
58 122  
59  
60 123

## 124 **Material and Methods**

### 125 *Cell culture*

126 HCT116 and HT29 human colon carcinoma cell lines were purchased from the  
127 American Type Culture Collection (ATCC). HT29 cell line (ATCC n°HTB-38) is BRAF  
128 mutant (V600E) and has a wild-RAS status. HCT116 cell line (ATCC n°CCL-247) has a  
129 wild-RAF status and is K-RAS mutated on codon 13 exon 2 (G13D). Cells were  
130 cultured in McCoy's medium (Thermo Fisher) supplemented with 10% foetal bovine  
131 serum (FBS). They were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.  
132 Cetuximab and panitumumab were obtained from the pharmacy of Tours University  
133 Hospital. Dilutions of cetuximab and panitumumab were prepared in phosphate  
134 buffer saline (PBS 1x). Cells were incubated with either cetuximab or panitumumab,  
135 two anti-EGFR antibodies (100µg/ml), or with buffer without antibody, twenty-four  
136 hours after plating. Regarding the literature, this dose corresponds to a very significant  
137 effect on viability of anti-EGFR sensitive-colorectal cancer cells [23]. These experiments  
138 were replicated 4 times for each condition (miRnome replicates).

139

### 140 *RNA extraction and expression profiling*

141 Total RNA was extracted from cells twenty-four hours after treatment using  
142 NucleoSpin miRNA kit according to the manufacturer's protocol (Macherey Nagel).  
143 Total RNA concentration and purity were measured using NanoDrop  
144 spectrophotometer (Thermo Fisher). Reverse transcription was done using miRCURY  
145 LNA RT kit according to the manufacturer's protocol (Qiagen). RNA isolation and  
146 cDNA synthesis were controlled by known UniSp6 RNA spike-ins. MiRnome study  
147 was done by real-time polymerase chain reaction (PCR) from obtained cDNA using  
148 miRCURY SYBR Green PCR kit according to the manufacturer's protocol (Qiagen).  
149 We used PCR plates of 384 wells containing spikes of a commercially available 752  
150 panel of Human miRs (Qiagen. miRCURY LNA miRNA miRNome PCR Panels; ID:  
151 339322). 45 cycles of real-time quantitative PCR were done in a LightCycler®480  
152 (Roche). Data were analyzed using LightCycler®480 software obtaining raw numbers

1  
2  
3 153 of cycles to threshold (Ct) for each well. Each Ct for each well was normalized with Ct  
4  
5 154 of commonly used known control and stable miRs (hsa-miR-7-5p, hsa-miR-191-5p,  
6  
7 155 hsa-miR-103a-3p, hsa-let-7a-5p, hsa-miR-16-5p, hsa-miR-34b-3p). Synthetic spike-in  
8  
9 156 templates (UniSp6, U6snARN, UniSp3 IPC, cel-miR-39-3p, water), are used to control  
10  
11 157 amplification and calibration. These templates are used step-by-step to verify RNA  
12  
13 158 extraction, reverse transcription and RTq-PCR quality.  
14

15 159

### 16 17 160 *Bio-informatic tools*

18  
19  
20 161 We used bioinformatic tools to determine validated and suspected targets of  
21  
22 162 previously selected miRs. Five databases were used and chosen according to their  
23  
24 163 characteristics as described in scientific publications [24,25]. Targetscan (v7.2) and  
25  
26 164 DIANA-Tools with its microT-CDS extension (v5) were used as tools predicting  
27  
28 165 interaction between the studied miR and specific targets. Targetscan is more robust  
29  
30 166 with the best balance between specificity and sensibility in terms of prediction [25]. It  
31  
32 167 is also the most frequently updated database [24]. Each potential target is ranked  
33  
34 168 according to an interaction probability score based on the matching rate of the target  
35  
36 169 “seed region” and the studied miR. DIANA-Tools with its microT-CDS extension, as  
37  
38 170 well as using an interaction probability score, can take into account non-conserved and  
39  
40 171 rarer interference regions [24]. Three other databases were used to screen  
41  
42 172 experimentally validated miR-target interaction: miRTarBase (v7.0), miRWalk (v2.0)  
43  
44 173 and TarBase (v8.0) extension of DIANA-Tools. They were chosen because they contain  
45  
46 174 the most validated studies and are frequently updated. miRWalk already cross-  
47  
48 175 references data issued from other bases: PhenomiR, miR2Disease, miRTarBase and  
49  
50 176 HMDB (Human Metabolome Database). The miR we studied in these databases are  
51  
52 177 those preliminarily selected from the miRnome cell study. The targets screened are key  
53  
54 178 components of the EGFR pathway in colorectal cancer cells. We chose them by using  
55  
56 179 KEGG-pathway (Kyoto Encyclopedia of Genes and Genomes Pathway) [26].  
57  
58 180 Interactions between miR and their targets (miR-Tar) were confirmed when cross-  
59  
60 181 referenced on 4 out of 5 database analyzed. We non exhaustively reviewed scientific  
182 literature using Medline concerning the miR confirmed to interact with the EGFR

1  
2  
3 183 pathway in order to determine the scientific interest held on them. We used the  
4  
5 184 keywords [hsa-miR-XXX], [cancer] or [colorectal cancer].  
6  
7  
8 185

### 186 *Statistical analysis of miRnome data*

187 Normalized Ct ( $\Delta$ Ct) for tested miR were compared between treated conditions and  
188 corresponding controls. From these comparisons was calculated the differential  
189 expression coefficient (DEC) which is the ratio of the miR expression on studied cells  
190 and the one on the control group. Values of the DEC are included in the  $]0 ; + \infty[$   
191 interval. When DEC is higher than 2, miR expression is considered significantly  
192 increased. When DEC is lower than 0.5, miR expression is considered significantly  
193 decreased. Statistical significance of miR expression variability according to the tested  
194 condition was evaluated with Student's t-test using normalized Ct values. P values <  
195 0.05 were considered significant. Calculations were done using the online Qiagen®  
196 software (<https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo=off>).  
197 Vulcano charts were created using  
198 GraphPad Prism software (GraphPad, La Jolla, CA, United States).  
199

### 200 **Blood samples and miR validation**

201 MiR identified in the two-phase process were measured in serum samples collected  
202 from patients with mCRC treated by panitumumab or cetuximab. These samples are  
203 part of a declared biological collection ( ) and were in conformity with  
204 the . This study of  
205 feasibility was an ancillary study performed after the constitution of the declared  
206 biological collection.

207 In these samples, miR were extracted using NucleoSpin miRNA Plasma/Serum kit  
208 (Macherey-Nagel). After reverse transcription, the expression and stability of several  
209 miRs from published literature about CRC were tested as reference genes for gene  
210 expression normalization: i.e. miR-25-3p, miR-103-5p, miR-191-5p, miR-340-5p, miR-  
211 484, miR-520d-5p, miR-627-5p, miR-1228-3p and U6. We validated miR-484 and miR-  
212 340-5p as references using GeNorm and NormFinder algorithms. PCR analysis was

1  
2  
3 213 performed with miRCURY LNA miRNA PCR Assays (Qiagen). A 35 Ct-threshold was  
4  
5 214 considered the maximum Ct value above which there is no physiological relevancy.  
6  
7  
8 215

## 11 216 **Results**

### 13 217 *Influence of cetuximab and panitumumab on HCT116 miR expression*

16 218 We have investigated miR suspected to be involved in anti-EGFR mAb resistance. To  
17  
18 219 that purpose we have used HCT116 cell line on which cetuximab and panitumumab  
19  
20 220 have less cytotoxic effect. By using micro-array technology, we have measured  
21  
22 221 HCT116 expression of a panel of the most common 752 Human miR (miRnome,  
23  
24 222 Exiqon/Qiagen). Three HCT116 miRnoms were compared: naïve, cetuximab and  
25  
26 223 panitumumab exposed. For each comparison, we have identified miR which  
27  
28 224 expression varied statistically significantly ( $p < 0.05$ ). In total, 17 miR significantly vary  
29  
30 225 with a variable amplitude measured by the differential expression coefficient (DEC)  
31  
32 226 (Figures 1 and Tables 1). After cetuximab exposure 4 miR were identified and all were  
33  
34 227 upregulated (Table 1A). After panitumumab exposure 8 miR were identified, 2 were  
35  
36 228 upregulated and 6 downregulated (Table 1B). Comparing cetuximab to panitumumab  
37  
38 229 exposure 7 miR were identified, 4 were upregulated and 3 downregulated (Table 1C).  
39  
40 230 Of the 17 miR, 4 were upregulated with an at least 2-fold coefficient and 1 was  
41  
42 231 downregulated with an at least 2-fold coefficient.  
43  
44 232

### 45 233 *Bio-informatic analysis of miR interaction with EGFR pathway*

48 234 From the 17 miR identified in the miRnome comparison phase, we distinguished those  
49  
50 235 interacting with the EGFR pathway (Figure 2). We first selected the targets of interest  
51  
52 236 by using the KEGG-pathway informatic tool. We chose the following targets: EGFR,  
53  
54 237 (H/N/K)RAS, (B/A)RAF, MAP2K(1/2), ERK, PIK3R, PIK3(Ca/R), AKT, MTOR,  
55  
56 238 RPS6KB, PTEN, MYC, GRB2, SOS, JUN, FOS, BCL2, CCND1, SNX1, PDCD4, FOXO.  
57  
58 239 Then, five databases were chosen according to specified criteria after having consulted  
59  
60 240 scientific literature. Two databases concern predicted interactions between miR and

1  
2  
3 241 targets (TargetScan and microT-CDS of DIANA Tools). Three databases concern  
4  
5 242 confirmed interactions between miR and targets (MiRTarBase, MiRWalk, TarBase of  
6  
7 243 DIANA Tools). For each database, we screened potential interaction of the 17 miR with  
8  
9 244 the 26 targets. The interaction was considered a highly plausible when found on four  
10  
11 245 out of five databases. They are presented on Table 2.

12  
13 246

### 14 15 247 *Selection of five miR as potential actors of anti-EGFR mAb resistance*

16  
17  
18 248 Next, we sharpened the miR selection according to specific criteria to select the most  
19  
20 249 likely miR as actors of colorectal cancer cell resistance to anti-EGFR mAb. We used a  
21  
22 250 two-phase selection process (Figure 3). The first selection was based on three criteria:  
23  
24 251 1. a significant ( $p < 0.05$ ) variation of expression of the miR after treatment exposure;  
25  
26 252 2. A DEC higher than 2 or lower than 0.5; 3. a highly plausible miR-target interaction  
27  
28 253 according to the bioinformatic analysis. miR that met the three benchmarks were  
29  
30 254 automatically selected ( $n = 2$ ). The ones which did not significantly vary in expression  
31  
32 255 ( $n = 737$ ) and the ones which significantly vary but had a DEC between 2 and 0.5 and  
33  
34 256 no target of interest, were excluded ( $n = 8$ ). The residing miR ( $n = 7$ ) went through the  
35  
36 257 second phase of the selection process. It consisted in identifying miR significantly  
37  
38 258 differentially expressed at baseline between cell lines of different sensitivity towards  
39  
40 259 anti-EGFR mAbs: HCT116 and HT29. Selected miR were those with a significant ( $p <$   
41  
42 260 0.01) differential expression and a DEC higher than 5 or lower than 0.2. In total, from  
43  
44 261 the 752 miR issued from each miRnome (commercially available panels from Qiagen),  
45  
46 262 5 miR were selected: miR-95-3p, miR-139-5p, miR-145-5p, miR-429 and miR-1247-5p.  
47  
48 263 Their characteristics are described in Table 3.

### 49 264 ***Validation of miR expression in serum of patients***

50  
51 265 Among the above selected miRs, we validated their expression in serum of mCRC  
52  
53 266 patients from our biological collection. In this tumor *in situ* context, only miR-139-5p  
54  
55 267 and miR-145-5p were found expressed under a 35 Ct threshold (table 4). This suggests  
56  
57 268 that we and others should probably focus on the evaluation of these miRs in ancillary  
58  
59 269 or prospective clinical studies.

## 270 Discussion

271 Mechanisms of initiation and progression of colorectal cancer are numerous and  
272 complex. Resistance to anti-EGFR monoclonal antibodies in mCRC makes necessary  
273 the search of new predictive biomarkers of tumour response. Micro-RNA are post-  
274 transcriptional regulators involved in various carcinogenic processes and particularly  
275 on the EGFR pathway in CRC [14]. While few miR measured from tumour tissue have  
276 been associated with anti-EGFR mAb response in CRC, none from serum or plasma  
277 have been identified [27].

278  
279 Cetuximab and panitumumab induce significant transcriptomic modifications of miR  
280 expression in colorectal cancer cell lines. These variations of expression affect single  
281 stranded miR as well as families of miR. In total, from the panel of 752 most common  
282 miR, only 17 had a significant change in expression after the miRnome comparison on  
283 HCT116 cells: 4 after cetuximab exposure, 8 after panitumumab exposure and 7  
284 comparing the two mAbs. It is interesting to note that only the expression of miR-429  
285 significantly varied after both treatment exposure. This indicates its important  
286 involvement in the EGFR pathway. The two anti-EGFR mAbs seem to have relatively  
287 the same mechanisms of action on the EGF receptor [4]. It would have seemed rational  
288 to have had the same variation of expression in both treatment groups. These  
289 variations can be explained by narrow distinct mAb characteristics. The EGFR  
290 epitopes targeted by both antibodies are yet overlapping (on domain III of the  
291 receptor) but functionally different in terms of specific binding sites [28]. Competition  
292 between the mAbs and EGF depends on their binding epitopes on the receptor.  
293 Panitumumab and EGF epitopes overlap in two locations (D355 and K443), whereas  
294 cetuximab and EGF epitopes overlap in five locations (D355, Q408, H409, H443 and  
295 S468) [28]. Thus, cetuximab and panitumumab affinity competition with EGF for the  
296 receptor are different [6]. In that sense, both mAbs affinity for EGFR has been  
297 calculated, with dissociation constants of 0.39nM for cetuximab vs 0.05nM for  
298 panitumumab [29]. Even though our *in vitro* study did not involve tumor micro-  
299 environment and systemic influence, it must not be omitted that by their

1  
2  
3 300 immunoglobulin subtype differences, cetuximab (IgG1) and panitumumab (IgG2)  
4  
5 301 mobilize different anti-tumor immune activity *in vivo*. Unlike panitumumab,  
6  
7 302 cetuximab stimulates natural killer cells for ADCC (antibody-dependent cellular  
8  
9 303 cytotoxicity) then activating as a chain reaction additional immunogenic processes  
10  
11 304 contributing to antitumor activity [30]. Instead, panitumumab seems to induce  
12  
13 305 neutrophil-driven ADCC and monocytes activation, but these immunostimulatory  
14  
15 306 capabilities do not influence the anti-tumor activity [30]. Hence, even if cetuximab and  
16  
17 307 panitumumab have a comparable therapeutic effect in mCRC, their respective  
18  
19 308 underlying mechanisms of action are different, thus suggesting evidence for  
20  
21 309 differences in miR expression as shown in our study. Also, our results are different  
22  
23 310 than those obtained on the same cell line in other studies. In Ragusa *et al* study, from  
24  
25 311 the 667 screened miR on cell line HCT116, 16 significantly varied in expression after  
26  
27 312 24h of cetuximab exposure at 20µg/ml and none corresponded to the ones we  
28  
29 313 identified but this could highlight a dose-dependent effect [31]. As we expected,  
30  
31 314 baseline miRnome of HCT116 and HT29 are different with 112 miR significantly  
32  
33 315 different, 26 over-expressed and 100 under-expressed (supplementary data, Tables 4).  
34  
35 316 Using a panel of 752 most common miR in Human allows to limit the screening field,  
36  
37 317 but this might have restricted it, omitting certain information. A next-generation  
38  
39 318 genomic sequencing would probably give rich supplementary data completing the  
40  
41 319 transcriptomic study. Comparing these two exploratory technics would be an  
42  
43 320 interesting line of research.

44  
45 321  
46 322 Some of the previously identified miR interact with the EGFR pathway. Five databases  
47  
48 323 have been selected for the bioinformatic study of evaluating potential interaction  
49  
50 324 between the 17 miR and main actors of the EGFR pathway. Three databases gather  
51  
52 325 experimentally validated interactions and are regularly updated. Two others weight  
53  
54 326 an interaction probability score. Targetscan (v7.2) is the most robust of both with a best  
55  
56 327 balance between specificity and sensitivity of predicting interaction [32]. For a given  
57  
58 328 miR, each target is ranked according to an interaction score. This score measures the  
59  
60 329 complementarity of target mRNA sequence with the miR seed region, a 6-8 nucleotides

1  
2  
3 330 sequence near the 5'UTR miR extremity. Those two sequences need to have a perfect  
4  
5 331 complementarity in Human for the miR to regulate the mRNA expression. MicroT-  
6  
7 332 CDS (v5) takes into account non conserved and rarer sites being able to interfere in  
8  
9 333 miR-target interaction [24]. These five databases are commonly used in studies of miR  
10  
11 334 expression. We have chosen the restrictive criteria of needing at least four similar  
12  
13 335 results on databases to validate an interaction between a miR with its target in order  
14  
15 336 to optimize the probability. From the 17 identified miR which expressions are  
16  
17 337 influenced by anti-EGFR mAbs on cell line HCT116, only 6 interact with a main actor  
18  
19 338 of the EGFR pathway. Three of those (miR-429, miR-95-3p and miR-29a-3p) act on  
20  
21 339 elements promoting the pathway's signal, as well as others inhibiting the signal. Four  
22  
23 340 of those miR (miR-429, miR-139-5p, miR-95-3p and miR-145-5p) act on transcription  
24  
25 341 factors (MYC, JUN and CCND1). The latter can then regulate the expression of other  
26  
27 342 miR. These two elements suggest hazy retro control mechanisms complex to model. It  
28  
29 343 would be interesting to analyze the interaction between a target with a miR as well as  
30  
31 344 the study we have done. This could enlighten the influence of certain transcription  
32  
33 345 factors on the miRnome variation that we have described.

34 346

35  
36 347 Five miR have a high probability of interference on colorectal cells' response to  
37  
38 348 cetuximab and panitumumab. We have established a two-phase process tree with  
39  
40 349 precise and restrictive criteria. This allowed to restrict further study from a panel of  
41  
42 350 752 miR to only 5 miR. Two of those miR (miR-95-3p and miR-139-5p) have been  
43  
44 351 directly selected since their expression significantly varied ( $p < 0.05$ ) with an important  
45  
46 352 coefficient ( $DEC > 2$  or  $< 0.5$ ) and interacted on the EGFR pathway according to the  
47  
48 353 bioinformatic analysis. Two other miR (miR-429 and miR-145-5p) needed their  
49  
50 354 baseline expression to be compared between the two studied cell lines: HCT116 anti-  
51  
52 355 EGFR mAb resistant and HT29 anti-EGFR mAb sensitive. Their expressions were  
53  
54 356 significantly different with an important coefficient. Even though the two mAbs have  
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56 357 similar therapeutic mechanisms of both treatments, the expression of miR-1247-5p  
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58 358 significantly varied directly comparing cetuximab exposure to panitumumab  
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60 359 exposure on HCT116. Also, miR-1247-5p has no clear predicted target on EGFR

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3 360 pathway. Yet, other studies seem to identify MYCBP2 as target of this miR. On  
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5 361 hypermethylated colorectal cancer cell models (HCT116 and RKO), miR-1247 inhibits  
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7 362 MYCBP2 protein expression as well as c-MYC's [33]. In the same study, over  
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9 363 expression of MYCBP2 was significantly associated with a lower overall survival,  
10  
11 364 suggesting an anti-tumoral effect of miR-1247. On prostate cancer cell lines, miR-1247-  
12  
13 365 5p inhibits MYCBP2 expression *in vitro* after transfection of mimic-miR and antago-  
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15 366 miR. For this reason, miR-1247-5p has been selected as potentially influencing  
16  
17 367 colorectal cancer cells sensitivity to anti-EGFR mAbs.

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19 368 Interestingly, none of the miR we have identified are of those associated with the  
20  
21 369 efficacy of anti-EGFR mAb in previous clinical studies. Of the most studied miR in  
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23 370 recent clinical studies, miR-31 shows to be correlated with cetuximab and  
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25 371 panitumumab efficacy [34]. The most frequently matured sequence is miR-31-5p. Its  
26  
27 372 over expression in primary tumour tissue is significantly associated with reduced PFS  
28  
29 373 without impact on OS in mCRC patients treated by cetuximab or panitumumab [35].  
30  
31 374 Similarly, in small retrospective cohorts, miR-31-3p over expression is associated with  
32  
33 375 reduced PFS in mCRC patients treated by anti-EGFR mAbs [16-18]. In a larger study,  
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35 376 Pugh et al [36] evaluated miR-31-3p expression in patients with operable CRC liver  
36  
37 377 metastases enrolled in New EPOC study. In patients treated by chemotherapy  
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39 378 associated with cetuximab, low expression of miR-31-3p was significantly associated  
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41 379 with longer PFS. More recently, Laurent-Puig et al investigated the predictive role of  
42  
43 380 miR-31-3p in a FIRE-3 study (NCT00433927) population which compared two  
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45 381 treatment groups of chemotherapy plus cetuximab or bevacizumab [37]. Data shows  
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47 382 that patients with low miR-31-3p expressing tumours had greater benefit from  
48  
49 383 cetuximab than from bevacizumab. These studies suggest that miR-31 expression level  
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51 384 might be a useful biomarker for the selection of mCRC treatment. In our study, cell  
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53 385 line HCT116 significantly express less miR-31-3p than HT29 at baseline  
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55 386 (Supplementary Table 4). This can be explained by their respective BRAF and KRAS  
56  
57 387 different mutational status, as previously described in pancreatic cancer cell lines [38].  
58  
59 388 However, when exposed to anti-EGFR mAb, miR-31 expression is not significantly  
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389 influenced. This might be explained by the fact that tumour mechanisms of miR-31  
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expression are independent from extra-cellular EGFR inhibition. Indeed, previous

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3 391 studies have shown that RAS oncogenic pathway induces miR-31 host gene expression  
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5 392 [39].  
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7 393 For clinical purpose, non-invasive methods of biomarker detection need to be  
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9 394 developed. In our study, miRnome analysis allowed to measure variations of intra-  
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11 395 cellular miR expression after anti-EGFR mAb exposure. It was shown that miR  
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13 396 detected in blood have two origins: free-floating from cell lysis and exosomal secretion  
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15 397 [40]. It remains unclear in which proportions miR are represented [41,42]. Circulating  
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17 398 free-floating miR could be representative to intra-cellular expression, which we  
18  
19 399 measured. Yet, miR contained in secreted exosomes are different than those kept in  
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21 400 the parent cell [43]. By complex miR-sequence dependent and independent sorting  
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23 401 methods, specific miR are incorporated into exosomes according to the concerned cell  
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25 402 activity [43]. Also, some packaged pre-miR finish their maturation process in  
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27 403 exosomes [40]. Thus, global miR signatures in blood could not be representative to  
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29 404 intra-cellular expression [41]. In CRC, several studies have identified circulating serum  
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31 405 miR to be biomarkers for diagnostic or prognostic stratification [27]. Only few ones  
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33 406 have profiled exosomal miR derived from CRC patients [44-48]. None have explored  
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35 407 serum miR signature with anti-EGFR mAb resistance in mCRC. Our study of  
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37 408 feasibility, which confirms the expression of miR-139-5p and miR-145-5p in serum of  
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39 409 mCRC patients treated with cetuximab or panitumumab, is a foothold in that  
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41 410 direction. However, further investigations are needed to confirm that the expression  
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43 411 of these 5 miRs in human fluids is associated with anti-EGFR mAb resistance.

## 412 **Conclusion**

44  
45  
46 413 Combining a large initial transcriptional screening with a thorough bioinformatic  
47  
48 414 study allowed us to select five miR candidates for potentially participating in  
49  
50 415 colorectal cancer cells' resistance to anti-EGFR mAbs. Experimental confirmation  
51  
52 416 needs to be completed by us and others. The ending goal being to identify circulating  
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54 417 miR as new non-invasive predictive biomarkers for cetuximab and panitumumab  
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56 418 efficacy on patients treated for mCRC, ideally in prospective studies.

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420 **Acknowledgments**

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430 **Authors' contributions**

431 [Redacted]  
432 [Redacted]  
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434 [Redacted]  
435 [Redacted]  
436 [Redacted]  
437 [Redacted]

438 **Conflict-of-interest statement/disclosure**

439 [Redacted]  
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3 446 **Executive summary**

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5 447 **Background**

6  
7 448 - Resistance to anti-EGFR monoclonal antibodies (mAb) in metastatic colorectal  
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9 449 cancer (mCRC) is impairing patients' survival.

10  
11 450 - MicroRNAs (miR) are regulators of colorectal cancer evolution and numerous  
12  
13 451 studies have reported dysregulations of miR according to the mCRC prognosis and  
14  
15 452 therapeutic response.

16 453 **Experimental section**

17  
18 454 - We measured cetuximab and panitumumab effect on miR expression in CRC cells  
19  
20 455 in order to identify key-miR influencing anti-EGFR mAb resistance thorough  
21  
22 456 bioinformatics study. We validated their expression in serum of mCRC patients.

23  
24 457 **Results and conclusion**

25  
26 458 - We identified *in vitro* miR-95-3p, miR-139-5p, miR-145-5p, miR-429 and miR-1247-  
27  
28 459 5p.

29  
30 460 - We found a significant expression of miR-139-5p and miR-145-5p in serum of  
31  
32 461 mCRC patients treated by cetuximab or panitumumab.

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34 462 - These latter miR are possible candidates for being biomarkers or even therapeutic  
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36 463 targets in the optimisation of cetuximab or panitumumab treatment in mCRC.

37 464  
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39 465 **References**

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42 466 **Papers of special note have been highlighted as: • of interest; •• of considerable**  
43  
44 467 **interest**

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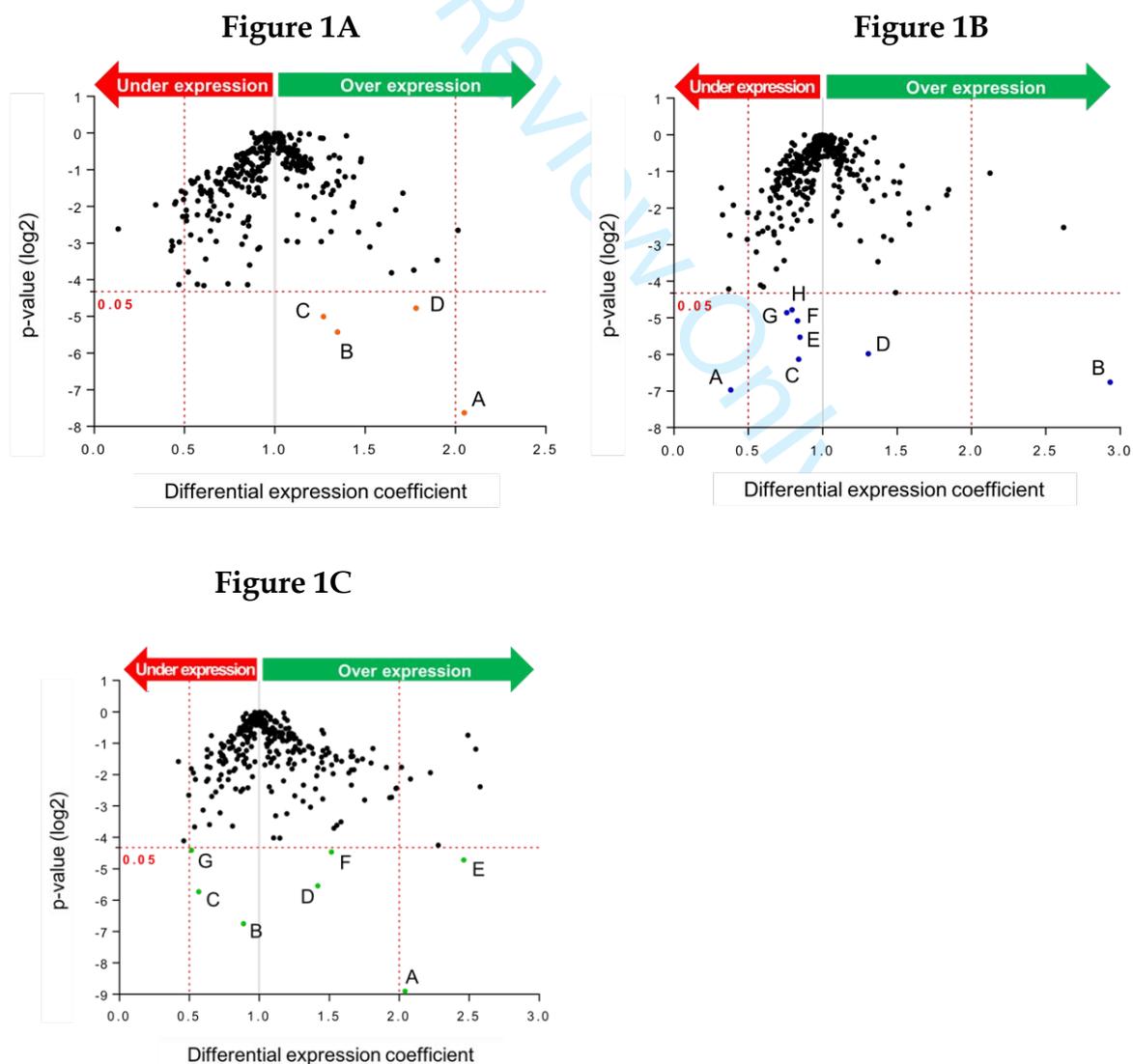
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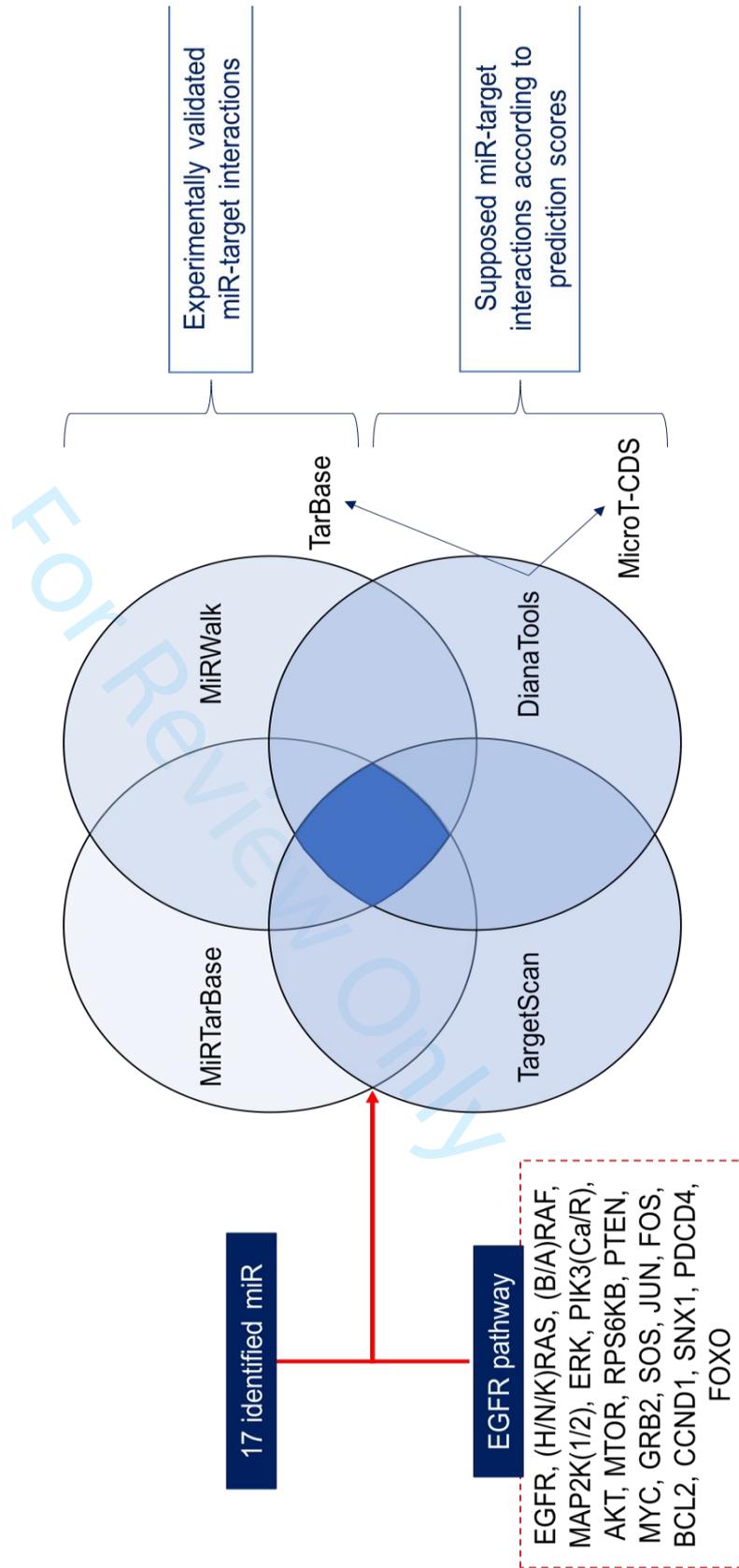
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## 616 Figure and legends

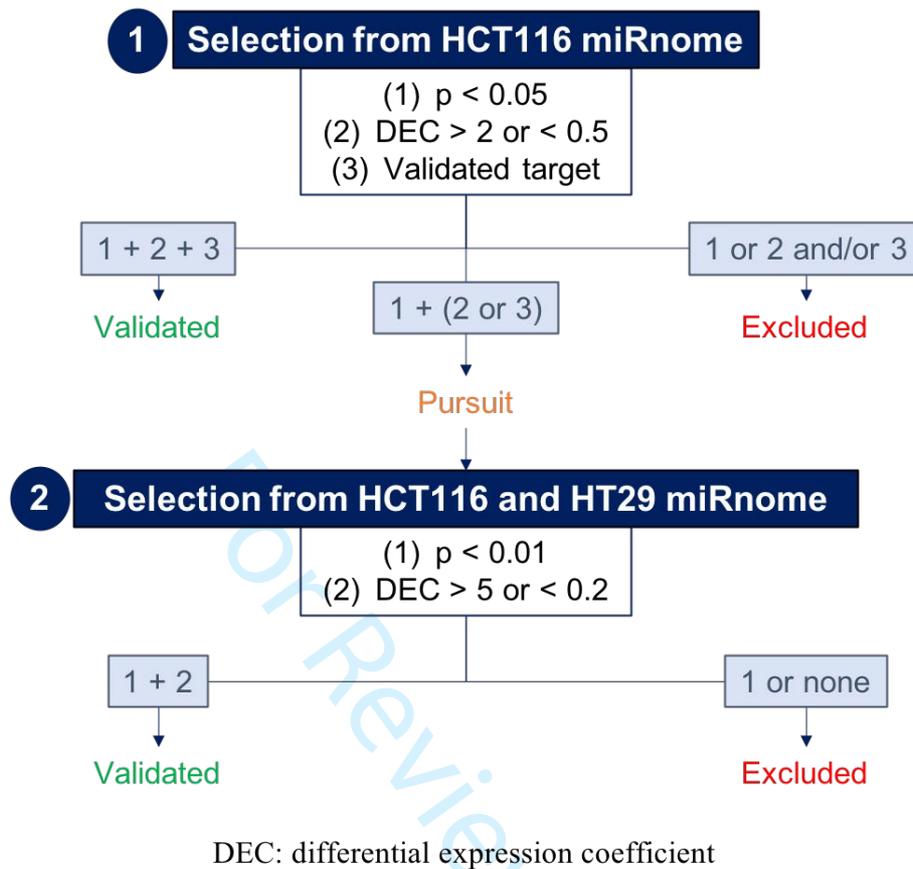
617 **Figure 1.** Volcano plots comparing the variation of expression of the 752 miR  
 618 (miRnome) in HCT116 cell line following cetuximab exposure (figure 1A),  
 619 panitumumab exposure (figure 1B) and in cetuximab-treated cells versus  
 620 panitumumab-treated cells (figure 1C). Student t-test using normalized Ct values was  
 621 used to evaluate statistical significance. Tests with p-values < 0.05 were considered  
 622 significant. The differential expression coefficient (DEC) is the ratio of the miR  
 623 expression in treated cells and the one in control cells. 4 differentially expressed miR  
 624 were identified and labeled as (A through D) in figure 1A ; 8 labeled as (A through H)  
 625 in figure 1B ; 7 labeled as (A through G) in figure 1C. All were represented in Table 1  
 626 with the equivalent label.



**Figure 2.** Bioinformatic tools used to identify miR interaction with the EGFR pathway. KEGG pathway was used to select targets of interest on the studied pathway. The 17 studied miR are those which expression varied significantly after treatment exposure on HCT116 cells (tables et figures 1). Databases gathering experimentally validated miR-target interactions are: miRTarBase, miRWalk and TarBase. Those gathering predicted interactions are: Targetscan and microT-CDS.



659 **Figure 3.** Two-phase process to select miR of anti-EGFR mAb resistance.



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662 **Table 1.** miR with significant variation of expression comparing non-treated cells with  
 663 cells exposed to cetuximab (table 1A), or to panitumumab (table 1B), and comparing  
 664 cells exposed to cetuximab with those exposed to panitumumab (table 1C). Student t-  
 665 test using normalized Ct values was used to evaluate statistical significance. Tests with  
 666 p-values  $< 0.05$  were considered significant. The differential expression coefficient  
 667 (DEC) is the ratio of the miR expression in treated cells and the one in control cells. 4  
 668 differentially expressed miR were identified and labeled as (A through D) in table 1A  
 669 ; 8 labeled as (A through H) in table 1B ; 7 labeled as (A through G) in table 1C. All  
 670 were represented in Figure 1 with the equivalent label. Those in bold have a DEC  $> 2$   
 671 or  $< 0.5$ .

**Table 1A**

Micro-RNA	DEC	p-value
<b>miR-627-5p (A)</b>	<b>2.05</b>	<b>0.0051</b>
miR-429 (B)	1.35	0.023
miR-378a-3p (C)	1.27	0.031
miR-501-5p (D)	1.78	0.037

672

**Table 1B**

Micro-RNA	DEC	p-value
<b>miR-139-5p (A)</b>	<b>0.383</b>	<b>0.0080</b>
<b>miR-95-3p (B)</b>	<b>2.93</b>	<b>0.0092</b>
miR-92a-3p (C)	0.839	0.014
miR-429 (D)	1.31	0.016
miR-505-3p (E)	0.847	0.022
miR-145-5p (F)	0.831	0.030
let-7g-5p (G)	0.758	0.034
miR-200a-3p (H)	0.794	0.036

673

**Table 1C**

Micro-RNA	DEC	p-value
<b>miR-140-3p (A)</b>	<b>2.04</b>	<b>0.0021</b>
miR-29a-3p (B)	0.887	0.0093
miR-627-5p (C)	0.567	0.019
miR-374a-5p (D)	1.42	0.021
<b>miR-1247-5p (E)</b>	<b>2.46</b>	<b>0.038</b>
miR-375 (F)	1.52	0.045
miR-421 (G)	0.515	0.047

674

675

676 **Table 2.** Identification of miR interacting with the EGFR pathway. The 17 miR are  
 677 those which expression varied significantly after treatment exposure on cell line  
 678 HCT116 (Tables 1). Selected targets of interest are the principal actors downstream the  
 679 EGFR. Validated miR-target interactions are those which are identified on 4 out of the  
 680 5 databases screened.

Micro-RNA	Target(s) of interest
miR-627-5p	0
miR-429	MYC, PTEN
miR-378a-3p	GRB2, MAPK1
miR-501-5p	0
miR-139-5p	JUN
miR-95-3p	SNX1, CCND1
miR-92a-3p	0
miR-505-3p	0
miR-145-5p	MYC
let-7g-5p	0
miR-200a-3p	0
miR-140-3p	0
miR-29a-3p	PIK3R1, PTEN
miR-374a-5p	0
miR-1247-5p	0
miR-375	0
miR-421	0

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**Table 3. Characteristics of the 5 miR selected with the two-phase process from the 752 initial miR.**

Micro-RNA	miR expression variation after anti-EGFR		Targets of interest		miR	expression	variation
	mAb exposure on HCT116 cells	<i>p-value</i>	DEC	DEC			
miR-429	0.016	1.35		MYC, PTEN	0.0016	6.29	
miR-95-3p	0.0092	2.93		CCND1, SNX1	0.12	0.562	
miR-145-5p	0.030	0.831		MYC	0.000022	0.0117	
miR-139-5p	0.0080	0.382		JUN	0.00089	0.20	
miR-1247-5p	0.038	2.46	0		0.22	0.19	

miR: micro-RNA; mAb: monoclonal antibodies; DEC: differential expression coefficient

<b>cetuximab-treated mCRC patients</b>			
	<b>Ct</b>	<b>reference miR-340-5p</b>	<b>reference miR-484</b>
<b>miR-95-3p</b>	≥ 35	30.6 +/- 0.9	28.2 +/- 0.5
<b>miR-139-5p</b>	30.9 +/- 1.0	30.6 +/- 0.7	28.3 +/- 1.0
<b>miR-145-5p</b>	28.4 +/- 0.8	30.5 +/- 1.3	27.5 +/- 0.9
<b>miR-429</b>	≥ 35	30.6 +/- 0.6	28.2 +/- 0.5
<b>miR-1247-5p</b>	not expressed		

<b>panitumumab-treated mCRC patients</b>			
	<b>Ct</b>	<b>reference miR-340-5p</b>	<b>reference miR-484</b>
<b>miR-95-3p</b>	≥ 35	29.3 +/- 1.5	26.9 +/- 1.4
<b>miR-139-5p</b>	29.3 +/- 1.0	29.7 +/- 1.6	26.9 +/- 1.5
<b>miR-145-5p</b>	27.4 +/- 1.2	29.3 +/- 1.9	26.1 +/- 1.5
<b>miR-429</b>	≥ 35	29.7 +/- 1.6	27 +/- 1.5
<b>miR-1247-5p</b>	not expressed		

**Table 4. Validation of miR expression in serum.** Mir expressions were evaluated in samples from 8 cetuximab and 8 panitumumab mCRC patients. Ct values are presented as means +/- SEM. A 35 Ct-threshold was considered the maximum Ct value above which there is no physiological relevance and a sample ≥ 40 Ct was considered as not expressed.

**Supplementary Table 4. Baseline miRnome comparison between HCT116 and HT29 cell lines.** Student t-test using normalized Ct values was used to evaluate statistical significance. Tests with p-values < 0.05 were considered significant. The differential expression coefficient (DEC) is the ratio of the miR expression in HCT116 cells and the one in HT29 cells. 26 miR were highly expressed (DEC > 2) and 18 of those were significantly differentially expressed (bold) in HCT116 cells compared to HT29 (Table 4A). 100 were less expressed (DEC < 0.5) and 57 of those were significantly differentially expressed (bold) in HCT116 cells compared to HT29 (Table 4B).

**Supplementary Table 4A.**

Micro-RNA	<i>p-value</i>	DEC
miR-194-5p	<b>0.003115</b>	<b>68.6094</b>
miR-192-5p	<b>&lt; 0.000001</b>	<b>65.5111</b>
miR-215-5p	<b>0.001083</b>	<b>61.6915</b>
miR-217	0.373902	<b>37.3232</b>
miR-338-3p	<b>0.00058</b>	<b>13.9052</b>
miR-375	<b>0.000687</b>	6.9339
miR-551b-3p	0.374293	6.8887
miR-429	<b>0.001579</b>	6.2926
miR-452-5p	<b>0.01689</b>	5.9808
miR-200b-5p	0.148333	5.6364
miR-10a-5p	<b>0.002009</b>	5.0545
miR-150-5p	<b>0.023155</b>	4.6403
miR-192-3p	<b>0.002565</b>	4.2864
miR-200b-3p	<b>0.000082</b>	4.0288
miR-10b-5p	<b>0.001817</b>	3.8557
miR-582-5p	<b>0.003039</b>	3.2661
miR-200a-3p	<b>0.000092</b>	3.1536
miR-615-3p	<b>0.029423</b>	3.1246
miR-598-3p	<b>0.004976</b>	2.964
miR-30b-5p	0.272737	2.4402
miR-663a	0.077984	2.4261
miR-196a-5p	<b>0.005045</b>	2.3086
miR-671-5p	0.389931	2.2979
let-7d-3p	0.274639	2.169
miR-18a-5p	<b>0.000498</b>	2.071
miR-874-3p	0.242043	2.0567

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Micro-RNA	<i>p</i> -value	DEC
miR-193b-3p	<b>0.000028</b>	0.4898
miR-135b-5p	0.200779	0.4864
miR-22-3p	0.054381	0.4853
miR-491-5p	0.106792	0.482
miR-744-3p	0.166962	0.4812
miR-590-3p	0.077807	0.4801
miR-1260a	0.055325	0.4735
miR-328-3p	<b>0.042997</b>	0.4672
miR-532-5p	<b>0.04356</b>	0.4645
miR-33b-3p	0.169822	0.4643
miR-624-5p	0.188462	0.4627
miR-423-5p	<b>0.031223</b>	0.4539
miR-23a-5p	<b>0.038376</b>	0.4518
miR-183-5p	<b>0.003713</b>	0.4487
miR-32-5p	0.160346	0.4394
miR-221-3p	<b>0.000903</b>	0.4384
miR-30c-5p	<b>0.004876</b>	0.4384
miR-339-5p	<b>0.041374</b>	0.4344
miR-151a-5p	0.312022	0.4231
miR-574-3p	<b>0.014907</b>	0.4225
miR-222-5p	0.130432	0.4132
miR-362-5p	<b>0.024581</b>	0.4101
miR-940	0.20696	0.4094
miR-193a-5p	0.165317	0.4054
miR-425-3p	<b>0.025438</b>	0.4043
miR-449a	0.120377	0.3976
miR-331-3p	<b>0.001028</b>	0.3924
miR-210-3p	<b>0.028789</b>	0.3924
miR-125b-1-3p	0.16956	0.3891
miR-130b-3p	<b>0.006162</b>	0.3861
miR-629-3p	0.179974	0.3819
miR-576-5p	0.074453	0.3774
miR-100-3p	0.249478	0.3767
miR-483-5p	0.147441	0.3763
miR-483-3p	<b>0.034668</b>	0.3732
miR-25-5p	0.053555	0.3706
miR-96-5p	<b>0.006493</b>	0.367
miR-31-3p	<b>0.000166</b>	0.352
miR-378a-3p	<b>0.007134</b>	0.352
miR-935	0.242083	0.3502
miR-7-1-3p	0.150725	0.3419
miR-28-3p	0.229604	0.3419
miR-182-5p	0.055866	0.3384
miR-21-5p	<b>0.009155</b>	0.3353
miR-877-3p	0.114327	0.3352
miR-629-5p	<b>0.018965</b>	0.3301
miR-29c-3p	<b>0.00114</b>	0.33
let-7g-3p	0.085362	0.326
let-7g-5p	<b>0.000261</b>	0.3239
miR-24-2-5p	0.124472	0.3204

Micro-RNA	<i>p</i> -value	DEC
miR-21-3p	<b>0.01299</b>	0.3071
miR-29b-3p	<b>0.042502</b>	0.3029
miR-551a	0.313293	0.3011
miR-29a-3p	<b>0.003448</b>	0.294
miR-140-5p	<b>0.043067</b>	0.2926
miR-502-3p	0.070659	0.2888
miR-191-5p	0.075584	0.282
miR-324-3p	<b>0.007786</b>	0.28
miR-132-3p	<b>0.027264</b>	0.2763
miR-193b-5p	<b>0.029623</b>	0.2679
miR-378a-5p	0.328121	0.2581
miR-149-5p	<b>0.033831</b>	0.2565
miR-152-3p	<b>0.006939</b>	0.2542
miR-362-3p	0.164082	0.2525
miR-766-3p	<b>0.002459</b>	0.2318
miR-9-3p	<b>0.037666</b>	0.227
let-7a-2-3p	0.151898	0.2222
miR-642a	<b>0.022725</b>	0.2122
miR-424-5p	<b>0.034786</b>	0.2108
miR-139-5p	<b>0.000889</b>	0.2065
miR-532-3p	0.251979	0.2056
miR-130b-5p	0.114341	0.2046
miR-660-5p	<b>0.001732</b>	0.1913
miR-1247-5p	0.223904	0.1909
miR-625-3p	<b>0.029385</b>	0.1769
miR-34c-3p	0.055881	0.172
miR-320a	<b>0.000035</b>	0.1708
miR-1296-5p	0.139268	0.167
miR-320c	0.05395	0.1651
miR-1237-3p	<b>0.019149</b>	0.16
miR-34a-3p	0.100265	0.1593
miR-744-5p	<b>0.002522</b>	0.1591
miR-320b	0.06319	0.1551
miR-455-3p	0.553475	0.1517
miR-29a-5p	0.139527	0.1492
miR-345-5p	<b>0.003718</b>	0.149
miR-1227-3p	<b>0.008133</b>	0.1424
miR-33a-5p	<b>0.04533</b>	0.1343
miR-342-3p	<b>0.002161</b>	0.1181
miR-505-3p	<b>&lt; 0.000001</b>	0.1125
miR-148a-3p	<b>0.00349</b>	<b>0.0793</b>
miR-153-3p	0.149335	<b>0.0779</b>
miR-320d	<b>0.0441</b>	<b>0.076</b>
miR-130a-3p	<b>0.000646</b>	<b>0.0536</b>
miR-126-3p	<b>0.000416</b>	<b>0.0466</b>
miR-502-5p	0.061029	<b>0.0466</b>
miR-34a-5p	<b>0.000386</b>	<b>0.0317</b>
miR-92b-3p	<b>0.015135</b>	<b>0.0164</b>
miR-9-5p	<b>0.042432</b>	<b>0.0164</b>
miR-145-5p	<b>0.000022</b>	<b>0.0117</b>
miR-125b-5p	<b>0.000017</b>	<b>0.0025</b>
miR-100-5p	<b>0.000628</b>	<b>0.0019</b>