Panitumumab and cetuximab affect differently miRNA expression in colorectal cancer cells

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Structured abstract

Background and aim

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

Results

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

Conclusion

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

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

Introduction

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

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

dependent cellular cytotoxicity due to its IgG2 nature [6]. It was demonstrated that in clinical practice, these two anti-EGFR mAbs have a comparable therapeutic efficacity in mCRC [7]. They can both be used as first line treatments if associated with a cytotoxic chemotherapy regimen, as well as in maintenance of mCRC. However, put aside their established overall survival increase, resistance to cetuximab and panitumumab is frequent in mCRC [8]. Biomarkers predicting anti-EGFR mAb efficacy in order to improve patients' selection for treatment, is a challenge which has justified numerous studies. To identify these biomarkers, understanding the mechanisms of resistance to these mAbs is essential. It appears that resistance to cetuximab and panitumumab is due to quantitative (over expression or amplification) and/or qualitative (mutations) anomalies in EGFR or to the independent or constitutive activation of intra-cellular downstream effectors (RAS, RAF, PI3K, PTEN). The only factors of resistance to cetuximab and panitumumab clearly demonstrated in clinical practice are the activating mutations of *KRAS* and *NRAS* genes [9]. They are at the origin of a RAS protein which is activated independently of EGFR status. Hence, blocking the EGFR by mAbs does not influence the pro-tumoral impact of the independently activated RAS pathway. *KRAS* mutations occurs in 40% of patients with mCRC [10]. However, of the wild-*RAS* patients who are treated by anti-EGFR mAbs, only 40% have a positive survival outcome [8]. It is therefore essential to identify new predictive biomarkers of anti-EGFR MAb efficacy in order to optimize mCRC treatment strategy.

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

causing deregulated miR production. Deregulated miR have an impact on diverse human diseases and especially on cancers. Mechanisms of miR deregulations in cancer cells are diverse [12]. Genetic alterations, preliminary transcriptional promoters' deregulations, epigenetic alterations and dysfunction in the enzymatic machinery of miR biogenesis, quantitatively and/or qualitatively modify miR influence [13]. As such, some miR are deregulated favoring pro-tumoral processes (onco-miR): pro-proliferation, anti-apoptotic, angiogenesis, epithelial-mesenchymal transition. Some of these onco-miR influence cardinal colorectal cancer pathways such as the EGFR-pathway, WNT/ßcatenin pathway, TGF-ß/Smad pathway or the MET pathways [14].

Some miR were shown to interact with the EGFR pathway in CRC but only few have been associated with the efficacy of anti-EGFR mAb in clinical practice [15]. All of those studied have been screened on primitive colorectal tumor tissue of patients treated by anti-EGFR mAb after several chemotherapy regimens. It seems that differential tumor expressions of miR-31-5p/3p, miR-592, miR-140-5p, miR-1224-5p, miR-181a, miR-302, miR-100, miR-125b and miR-7 are associated with survival and/or disease progression after cetuximab or panitumumab treatment [16-22]. However, the heterogeneity of these findings and the intricated elements influencing miR expression, makes it complicated to comprehend the underlying mechanisms of miR impact on anti-EGFR mAb efficacy in mCRC. Thanks to nowadays bio-informatic tools enriched by the growing interest of miR influence on cancer processes, this study aims at understanding the influence of cetuximab and panitumumab exposure on miR expression (MiRnome profiling) in colorectal cancer cells in order to identify those implicated in the EGFR pathway and resistance to anti-EGFR mAbs ; as well as to describe the possible differences in miR expression between these two anti-EGFR mAbs. Finally we aimed to validate the expression of these selected miRs in serum of patients with mCRC.

Material and Methods

Cell culture

HCT116 and HT29 human colon carcinoma cell lines were purchased from the American Type Culture Collection (ATCC). HT29 cell line (ATCC n°HTB-38) is BRAF mutant (V600E) and has a wild-RAS status. HCT116 cell line (ATCC n°CCL-247) has a wild-RAF status and is K-RAS mutated on codon 13 exon 2 (G13D). Cells were cultured in McCoy's medium (Thermo Fisher) supplemented with 10% foetal bovine serum (FBS). They were maintained at 37°C in a humidified atmosphere of 5% CO2. Cetuximab and panitumumab were obtained from the pharmacy of Tours University Hospital. Dilutions of cetuximab and panitumumab were prepared in phosphate buffer saline (PBS 1x). Cells were incubated with either cetuximab or panitumumab, two anti-EGFR antibodies (100µg/ml), or with buffer without antibody, twenty-four hours after plating. Regarding the literature, this dose corresponds to a very significant effect on viability of anti-EGFR sensitive-colorectal cancer cells [23]. These experiments were replicated 4 times for each condition (miRnome replicates).

RNA extraction and expression profiling

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

of cycles to threshold (Ct) for each well. Each Ct for each well was normalized with Ct of commonly used known control and stable miRs (hsa-miR-7-5p, hsa-miR-191-5p, hsa-miR-103a-3p, hsa-let-7a-5p, hsa-miR-16-5p, hsa-miR-34b-3p). Synthetic spike-in templates (UniSp6, U6snARN, UniSp3 IPC, cel-miR-39-3p, water), are used to control amplification and calibration. These templates are used step-by-step to verify RNA extraction, reverse transcription and RTq-PCR quality.

Bio-informatic tools

We used bioinformatic tools to determine validated and suspected targets of previously selected miRs. Five databases were used and chosen according to their characteristics as described in scientific publications [24,25]. Targetscan (v7.2) and DIANA-Tools with its microT-CDS extension (v5) were used as tools predicting interaction between the studied miR and specific targets. Targetscan is more robust with the best balance between specificity and sensibility in terms of prediction [25]. It is also the most frequently updated database [24]. Each potential target is ranked according to an interaction probability score based on the matching rate of the target "seed region" and the studied miR. DIANA-Tools with its microT-CDS extension, as well as using an interaction probability score, can take into account non-conserved and rarer interference regions [24]. Three other databases were used to screen experimentally validated miR-target interaction: miRTarBase (v7.0), miRWalk (v2.0) and TarBase (v8.0) extension of DIANA-Tools. They were chosen because they contain the most validated studies and are frequently updated. miRWalk already cross-references data issued from other bases: PhenomiR, miR2Disease, miRTarBase and HMDB (Human Metabolome Database). The miR we studied in these databases are those preliminarily selected from the miRnome cell study. The targets screened are key components of the EGFR pathway in colorectal cancer cells. We chose them by using KEGG-pathway (Kyoto Encyclopedia of Genes and Genomes Pathway) [26]. Interactions between miR and their targets (miR-Tar) were confirmed when cross-referenced on 4 out of 5 database analyzed. We non exhaustively reviewed scientific literature using Medline concerning the miR confirmed to interact with the EGFR

pathway in order to determine the scientific interest held on them. We used the keywords [hsa-miR-XXX], [cancer] or [colorectal cancer].

Statistical analysis of miRnome data

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

Blood samples and miR validation

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

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

performed with miRCURY LNA miRNA PCR Assays (Qiagen). A 35 Ct-threshold was considered the maximum Ct value above which there is no physiological relevancy.

Results

Influence of cetuximab and panitumumab on HCT116 miR expression

We have investigated miR suspected to be involved in anti-EGFR mAb resistance. To that purpose we have used HCT116 cell line on which cetuximab and panitumumab have less cytotoxic effect. By using micro-array technology, we have measured HCT116 expression of a panel of the most common 752 Human miR (miRnome, Exiqon/Qiagen). Three HCT116 miRnomes were compared: naïve, cetuximab and panitumumab exposed. For each comparison, we have identified miR which expression varied statistically significantly ($p < 0.05$). In total, 17 miR significantly vary with a variable amplitude measured by the differential expression coefficient (DEC) (Figures 1 and Tables 1). After cetuximab exposure 4 miR were identified and all were upregulated (Table 1A). After panitumumab exposure 8 miR were identified, 2 were upregulated and 6 downregulated (Table 1B). Comparing cetuximab to panitumumab exposure 7 miR were identified, 4 were upregulated and 3 downregulated (Table 1C). Of the 17 miR, 4 were upregulated with an at least 2-fold coefficient and 1 was downregulated with an at least 2-fold coefficient.

Bio-informatic analysis of miR interaction with EGFR pathway

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

targets (TargetScan and microT-CDS of DIANA Tools). Three databases concern confirmed interactions between miR and targets (MiRTarBase, MiRWalk, TarBase of DIANA Tools). For each database, we screened potential interaction of the 17 miR with the 26 targets. The interaction was considered a highly plausible when found on four out of five databases. They are presented on Table 2.

Selection of five miR as potential actors of anti-EGFR mAb resistance

Next, we sharpened the miR selection according to specific criteria to select the most likely miR as actors of colorectal cancer cell resistance to anti-EGFR mAb. We used a two-phase selection process (Figure 3). The first selection was based on three criteria: 1. a significant ($p < 0.05$) variation of expression of the miR after treatment exposure; 2. A DEC higher than 2 or lower than 0.5; 3. a highly plausible miR-target interaction according to the bioinformatic analysis. miR that met the three benchmarks were automatically selected (n = 2). The ones which did not significantly vary in expression (n = 737) and the ones which significantly vary but had a DEC between 2 and 0.5 and no target of interest, were excluded (n = 8). The residing miR (n = 7) went through the second phase of the selection process. It consisted in identifying miR significantly differentially expressed at baseline between cell lines of different sensitivity towards anti-EGFR mAbs: HCT116 and HT29. Selected miR were those with a significant ($p < 0.01$) differential expression and a DEC higher than 5 or lower than 0.2. In total, from the 752 miR issued from each miRnome (commercially available panels from Qiagen), 5 miR were selected: miR-95-3p, miR-139-5p, miR-145-5p, miR-429 and miR-1247-5p. Their characteristics are described in Table 3.

Validation of miR expression in serum of patients

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

Discussion

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

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

immunoglobulin subtype differences, cetuximab (IgG1) and panitumumab (IgG2) mobilize different anti-tumor immune activity *in vivo*. Unlike panitumumab, cetuximab stimulates natural killer cells for ADCC (antibody-dependent cellular cytotoxicity) then activating as a chain reaction additional immunogenic processes contributing to antitumor activity [30]. Instead, panitumumab seems to induce neutrophil-driven ADCC and monocytes activation, but these immunostimulatory capabilities do not influence the anti-tumor activity [30]. Hence, even if cetuximab and panitumumab have a comparable therapeutic effect in mCRC, their respective underlying mechanisms of action are different, thus suggesting evidence for differences in miR expression as shown in our study. Also, our results are different than those obtained on the same cell line in other studies. In Ragusa *et al* study, from the 667 screened miR on cell line HCT116, 16 significantly varied in expression after 24h of cetuximab exposure at 20µg/ml and none corresponded to the ones we identified but this could highlight a dose-dependent effect [31]. As we expected, baseline miRnome of HCT116 and HT29 are different with 112 miR significatively different, 26 over-expressed and 100 under-expressed (supplementary data, Tables 4). Using a panel of 752 most common miR in Human allows to limit the screening field, but this might have restricted it, omitting certain information. A next-generation genomic sequencing would probably give rich supplementary data completing the transcriptomic study. Comparing these two exploratory technics would be an interesting line of research.

Some of the previously identified miR interact with the EGFR pathway. Five databases have been selected for the bioinformatic study of evaluating potential interaction between the 17 miR and main actors of the EGFR pathway. Three databases gather experimentally validated interactions and are regularly updated. Two others weight an interaction probability score. Targetscan (v7.2) is the most robust of both with a best balance between specificity and sensitivity of predicting interaction [32]. For a given miR, each target is ranked according to an interaction score. This score measures the complementarity of target mRNA sequence with the miR seed region, a 6-8 nucleotides

sequence near the 5'UTR miR extremity. Those two sequences need to have a perfect complementarity in Human for the miR to regulate the mRNA expression. MicroT-CDS (v5) takes into account non conserved and rarer sites being able to interfere in miR-target interaction [24]. These five databases are commonly used in studies of miR expression. We have chosen the restrictive criteria of needing at least four similar results on databases to validate an interaction between a miR with its target in order to optimize the probability. From the 17 identified miR which expressions are influenced by anti-EGFR mAbs on cell line HCT116, only 6 interact with a main actor of the EGFR pathway. Three of those (miR-429, miR-95-3p and miR-29a-3p) act on elements promoting the pathway's signal, as well as others inhibiting the signal. Four of those miR (miR-429, miR-139-5p, miR-95-3p and miR-145-5p) act on transcription factors (MYC, JUN and CCND1). The latter can then regulate the expression of other miR. These two elements suggest hazy retro control mechanisms complex to model. It would be interesting to analyze the interaction between a target with a miR as well as the study we have done. This could enlighten the influence of certain transcription factors on the miRnome variation that we have described.

Five miR have a high probability of interference on colorectal cells' response to cetuximab and panitumumab. We have established a two-phase process tree with precise and restrictive criteria. This allowed to restrict further study from a panel of 752 miR to only 5 miR. Two of those miR (miR-95-3p and miR-139-5p) have been directly selected since their expression significantly varied ($p < 0.05$) with an important coefficient (DEC > 2 or < 0.5) and interacted on the EGFR pathway according to the bioinformatic analysis. Two other miR (miR-429 and miR-145-5p) needed their baseline expression to be compared between the two studied cell lines: HCT116 anti-EGFR mAb resistant and HT29 anti-EGFR mAb sensitive. Their expressions were significantly different with an important coefficient. Even though the two mAbs have similar therapeutic mechanisms of both treatments, the expression of miR-1247-5p significantly varied directly comparing cetuximab exposure to panitumumab exposure on HCT116. Also, miR-1247-5p has no clear predicted target on EGFR

pathway. Yet, other studies seem to identify MYCBP2 as target of this miR. On hypermethylated colorectal cancer cell models (HCT116 and RKO), miR-1247 inhibits MYCBP2 protein expression as well as c-MYC's [33]. In the same study, over expression of MYCBP2 was significatively associated with a lower overall survival, suggesting an anti-tumoral effect of miR-1247. On prostate cancer cell lines, miR-1247-5p inhibits MYCBP2 expression *in vitro* after transfection of mimic-miR and antago-miR. For this reason, miR-1247-5p has been selected as potentially influencing colorectal cancer cells sensitivity to anti-EGFR mAbs.

Interestingly, none of the miR we have identified are of those associated with the efficacy of anti-EGFR mAb in previous clinical studies. Of the most studied miR in recent clinical studies, miR-31 shows to be correlated with cetuximab and panitumumab efficacy [34]. The most frequently matured sequence is miR-31-5p. Its over expression in primary tumour tissue is significantly associated with reduced PFS without impact on OS in mCRC patients treated by cetuximab or panitumumab [35]. Similarly, in small retrospective cohorts, miR-31-3p over expression is associated with reduced PFS in mCRC patients treated by anti-EGFR mAbs [16-18]. In a larger study, Pugh et al [36] evaluated miR-31-3p expression in patients with operable CRC liver metastases enrolled in New EPOC study. In patients treated by chemotherapy associated with cetuximab, low expression of miR-31-3p was significantly associated with longer PFS. More recently, Laurent-Puig et al investigated the predictive role of miR-31-3p in a FIRE-3 study (NCT00433927) population which compared two treatment groups of chemotherapy plus cetuximab or bevacizumab [37]. Data shows that patients with low miR-31-3p expressing tumours had greater benefit from cetuximab than from bevacizumab. These studies suggest that miR-31 expression level might be a useful biomarker for the selection of mCRC treatment. In our study, cell line HCT116 significantly express less miR-31-3p than HT29 at baseline (Supplementary Table 4). This can be explained by their respective BRAF and KRAS different mutational status, as previously described in pancreatic cancer cell lines [38]. However, when exposed to anti-EGFR mAb, miR-31 expression is not significantly influenced. This might be explained by the fact that tumour mechanisms of miR-31 expression are independent from extra-cellular EGFR inhibition. Indeed, previous

studies have shown that RAS oncogenic pathway induces miR-31 host gene expression [39].

For clinical purpose, non-invasive methods of biomarker detection need to be developed. In our study, miRnome analysis allowed to measure variations of intra-cellular miR expression after anti-EGFR mAb exposure. It was shown that miR detected in blood have two origins: free-floating from cell lysis and exosomal secretion [40]. It remains unclear in which proportions miR are represented [41,42]. Circulating free-floating miR could be representative to intra-cellular expression, which we measured. Yet, miR contained in secreted exosomes are different than those kept in the parent cell [43]. By complex miR-sequence dependent and independent sorting methods, specific miR are incorporated into exosomes according to the concerned cell activity [43]. Also, some packaged pre-miR finish their maturation process in exosomes [40]. Thus, global miR signatures in blood could not be representative to intra-cellular expression [41]. In CRC, several studies have identified circulating serum miR to be biomarkers for diagnostic or prognostic stratification [27]. Only few ones have profiled exosomal miR derived from CRC patients [44-48]. None have explored serum miR signature with anti-EGFR mAb resistance in mCRC. Our study of feasibility, which confirms the expression of miR-139-5p and miR-145-5p in serum of mCRC patients treated with cetuximab or panitumumab, is a foothold in that direction. However, further investigations are needed to confirm that the expression of these 5 miRs in human fluids is associated with anti-EGFR mAb resistance.

Conclusion

Combining a large initial transcriptional screening with a thorough bioinformatic study allowed us to select five miR candidates for potentially participating in colorectal cancer cells' resistance to anti-EGFR mAbs. Experimental confirmation needs to be completed by us and others. The ending goal being to identify circulating miR as new non-invasive predictive biomarkers for cetuximab and panitumumab efficacy on patients treated for mCRC, ideally in prospective studies.

Acknowledgments

[redacted]

Authors' contributions

[redacted]

Conflict-of-interest statement/disclosure

[redacted]

Executive summary

Background

- Resistance to anti-EGFR monoclonal antibodies (mAb) in metastatic colorectal cancer (mCRC) is impairing patients' survival.
- MicroRNAs (miR) are regulators of colorectal cancer evolution and numerous studies have reported dysregulations of miR according to the mCRC prognosis and therapeutic response.

Experimental section

- We measured cetuximab and panitumumab effect on miR expression in CRC cells in order to identify key-miR influencing anti-EGFR mAb resistance thorough bioinformatics study. We validated their expression in serum of mCRC patients.

Results and conclusion

- We identified *in vitro* miR-95-3p, miR-139-5p, miR-145-5p, miR-429 and miR-1247-5p.
- We found a significant expression of miR-139-5p and miR-145-5p in serum of mCRC patients treated by cetuximab or panitumumab.
- These latter miR are possible candidates for being biomarkers or even therapeutic targets in the optimisation of cetuximab or panitumumab treatment in mCRC.

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Figure and legends

Figure 1. Volcano plots comparing the variation of expression of the 752 miR (miRnome) in HCT116 cell line following cetuximab exposure (figure 1A), panitumumab exposure (figure 1B) and in cetuximab-treated cells versus panitumumab-treated cells (figure 1C). 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 treated cells and the one in control cells. 4 differentially expressed miR were identified and labeled as (A through D) in figure 1A ; 8 labeled as (A through H) in figure 1B ; 7 labeled as (A through G) in figure 1C. All were represented in Table 1 with the equivalent label.

Figure 1A

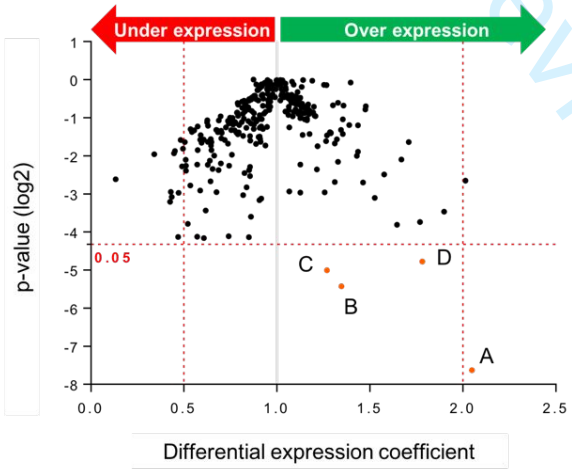


Figure 1B

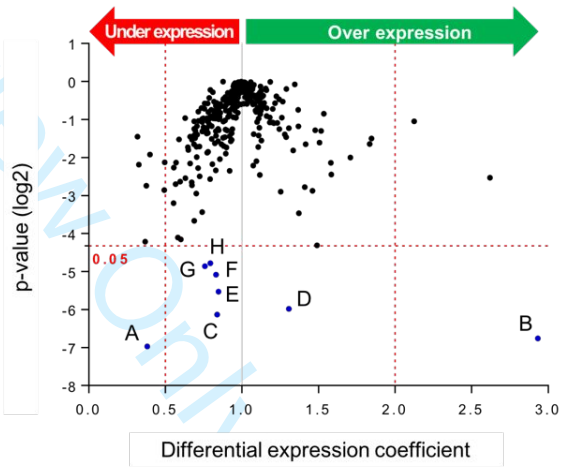
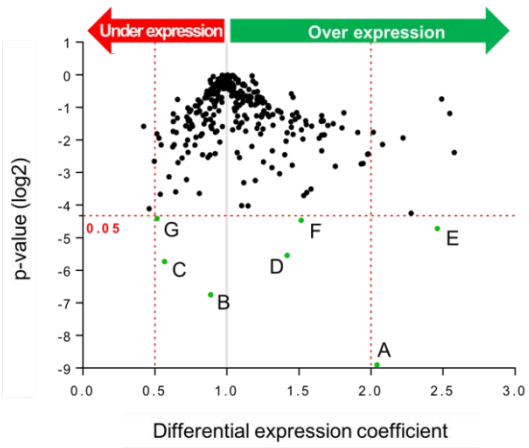


Figure 1C



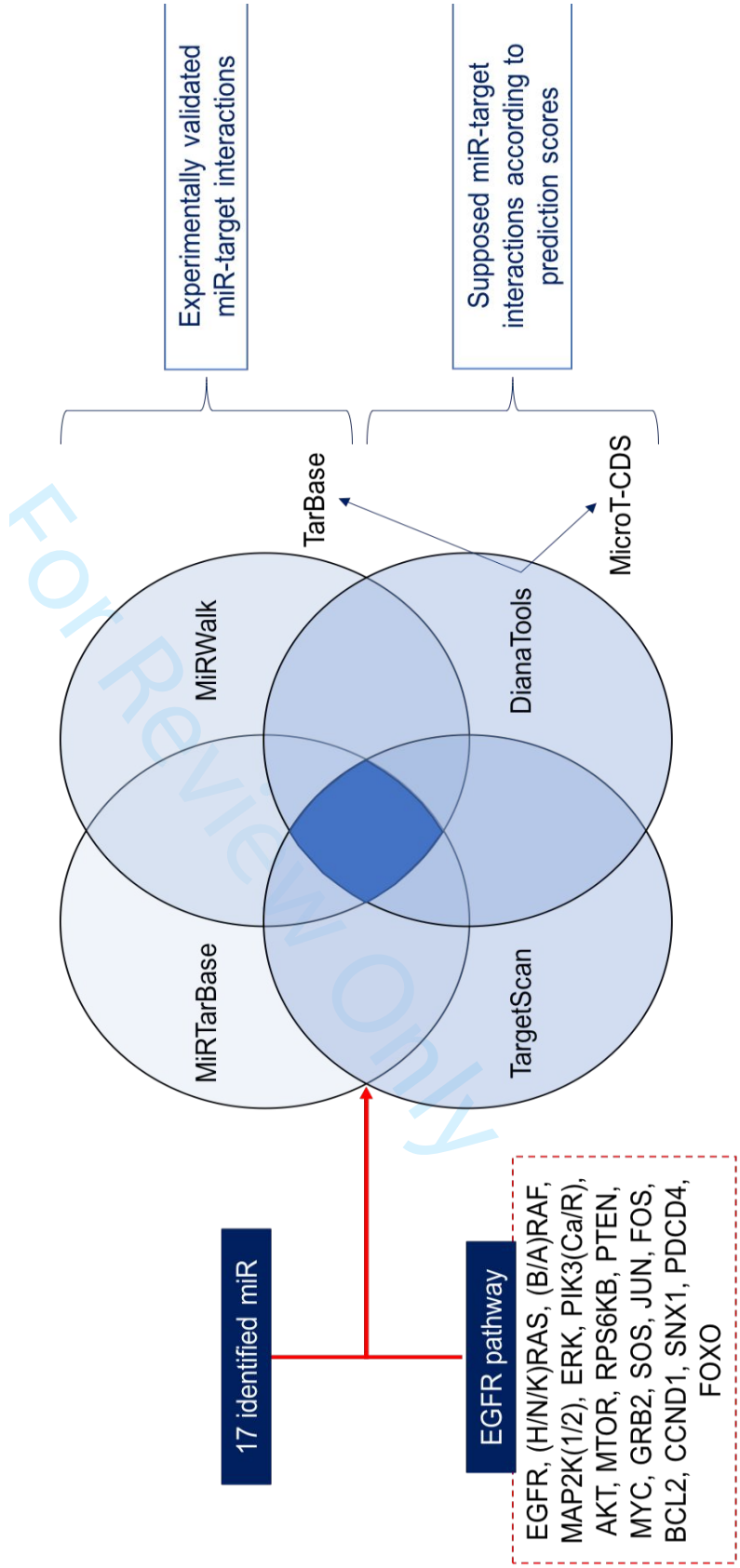
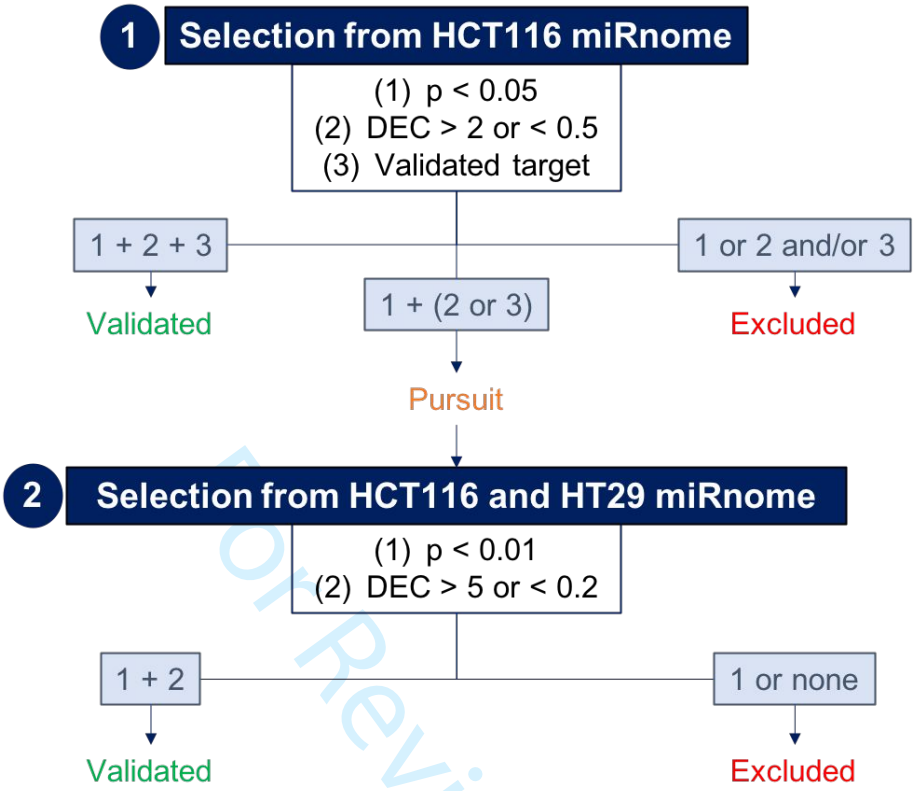


Figure 2. Bioinformatic tools used to identify miR interaction with the EGFR pathway. KEGG pathway was used to select targets of interest on the studied pathway. The 17 studied miR are those which expression varied significantly after treatment exposure on HCT116 cells (tables et figures 1). Databases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

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



DEC: differential expression coefficient

Table 1. miR with significant variation of expression comparing non-treated cells with cells exposed to cetuximab (table 1A), or to panitumumab (table 1B), and comparing cells exposed to cetuximab with those exposed to panitumumab (table 1C). 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 treated cells and the one in control cells. 4 differentially expressed miR were identified and labeled as (A through D) in table 1A ; 8 labeled as (A through H) in table 1B ; 7 labeled as (A through G) in table 1C. All were represented in Figure 1 with the equivalent label. Those in bold have a DEC > 2 or < 0.5.

Table 1A

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

Table 1B

Micro-RNA	DEC	p-value
miR-139-5p (A)	**0.383**	**0.0080**
miR-95-3p (B)	**2.93**	**0.0092**
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

Table 1C

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

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

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 mAb exposure on HCT116 cells		Targets of interest	Baseline miR expression variation comparing HCT116 and HT29 cells	
	p-value	DEC		*p-value*	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

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

	panitumumab-treated mCRC patients		
	Ct	**reference miR-340-5p**	**reference miR-484**
miR-95-3p	≥ 35	29.3 +/- 1.5	26.9 +/- 1.4
miR-139-5p	29.3 +/- 1.0	29.7 +/- 1.6	26.9 +/- 1.5
miR-145-5p	27.4 +/- 1.2	29.3 +/- 1.9	26.1 +/- 1.5
miR-429	≥ 35	29.7 +/- 1.6	27 +/- 1.5
miR-1247-5p	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	*p-value*	DEC
miR-194-5p	**0.003115**	**68.6094**
miR-192-5p	**< 0.000001**	**65.5111**
miR-215-5p	**0.001083**	**61.6915**
miR-217	0.373902	**37.3232**
miR-338-3p	**0.00058**	**13.9052**
miR-375	**0.000687**	6.9339
miR-551b-3p	0.374293	6.8887
miR-429	**0.001579**	6.2926
miR-452-5p	**0.01689**	5.9808
miR-200b-5p	0.148333	5.6364
miR-10a-5p	**0.002009**	5.0545
miR-150-5p	**0.023155**	4.6403
miR-192-3p	**0.002565**	4.2864
miR-200b-3p	**0.000082**	4.0288
miR-10b-5p	**0.001817**	3.8557
miR-582-5p	**0.003039**	3.2661
miR-200a-3p	**0.000092**	3.1536
miR-615-3p	**0.029423**	3.1246
miR-598-3p	**0.004976**	2.964
miR-30b-5p	0.272737	2.4402
miR-663a	0.077984	2.4261
miR-196a-5p	**0.005045**	2.3086
miR-671-5p	0.389931	2.2979
let-7d-3p	0.274639	2.169
miR-18a-5p	**0.000498**	2.071
miR-874-3p	0.242043	2.0567

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720 **Supplementary Tables 4B**

Micro-RNA	*p-value*	DEC
miR-193b-3p	**0.000028**	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	**0.042997**	0.4672
miR-532-5p	**0.04356**	0.4645
miR-33b-3p	0.169822	0.4643
miR-624-5p	0.188462	0.4627
miR-423-5p	**0.031223**	0.4539
miR-23a-5p	**0.038376**	0.4518
miR-183-5p	**0.003713**	0.4487
miR-32-5p	0.160346	0.4394
miR-221-3p	**0.000903**	0.4384
miR-30c-5p	**0.004876**	0.4384
miR-339-5p	**0.041374**	0.4344
miR-151a-5p	0.312022	0.4231
miR-574-3p	**0.014907**	0.4225
miR-222-5p	0.130432	0.4132
miR-362-5p	**0.024581**	0.4101
miR-940	0.20696	0.4094
miR-193a-5p	0.165317	0.4054
miR-425-3p	**0.025438**	0.4043
miR-449a	0.120377	0.3976
miR-331-3p	**0.001028**	0.3924
miR-210-3p	**0.028789**	0.3924
miR-125b-1-3p	0.16956	0.3891
miR-130b-3p	**0.006162**	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	**0.034668**	0.3732
miR-25-5p	0.053555	0.3706
miR-96-5p	**0.006493**	0.367
miR-31-3p	**0.000166**	0.352
miR-378a-3p	**0.007134**	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	**0.009155**	0.3353
miR-877-3p	0.114327	0.3352
miR-629-5p	**0.018965**	0.3301
miR-29c-3p	**0.00114**	0.33
let-7g-3p	0.085362	0.326
let-7g-5p	**0.000261**	0.3239
miR-24-2-5p	0.124472	0.3204

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

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