microRNA Gene Expression Profiling of Colorectal Cell Lines: Prediction of Drug Sensitivity and Correlation to Mutation Status

Sarah Runswick, Susie Weston, Nikki Crow and Susan Lovick

DM CIRA AstraZeneca, Alderley Park, Macclesfield. Cheshire, UK

Background

Gene expression profiles for a panel microRNAs (miRNAs) for colorectal cancer (CRC) cell lines have been generated. Data analyses have been performed to determine if:

1. real time RT-PCR of miRNA using Low Taqman Density Arrays (TLDA, Applied Biosystems platform) is a robust method to measure miRNA gene expression
2. the potential for miRNA gene expression profiling to be used to predict sensitivity of resistance to oncology drugs
3. linkage between miRNA gene expression and mutational status for CRC cell lines

Introduction

56 colorectal cell lines have been profiled against a panel of miRNAs. These cell lines are included in the extended CRC cell line panel which have complementary pharmacology data, Affymetrix gene expression data and genetic data (CLIMB database). Cell lines were predominantly isolated from patients with Dukes stage B-D disease and there are two lines from stage A (SW1116 & C106).

Included in the panel are the following paired cell lines:

- VACO4A / VACO4S
- Colo201 / Colo206
- DLD1 / HCT15CV
- HT29 / WIDR
- LS174T / LS180
- SW480 / SW620

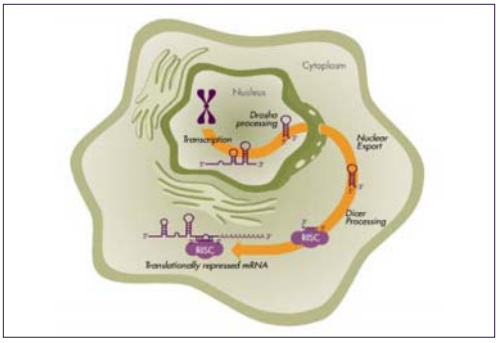


Figure 1: pre-miRNA are processed by Drosha and Pasha in the nuclear envelope, and by Dicer in the cytoplasm. The mature microRNA are incorporated into the RISC complex, and target mRNA.

microRNAs are non-coding, single-stranded endogenous RNAs, 18-23 nucleotides long. They are post-transcriptional regulators that bind to homologous sequences in the 3' untranslated region of mRNA. They either silence the mRNA or initiate its cleavage, depending on the degree of homology. Silenced mRNA accumulate in the cytoplasm as processing bodies.

miRNA expression is deregulated at the initiation, proliferation and vascularisation of most stages of cancer.

Materials and Methods

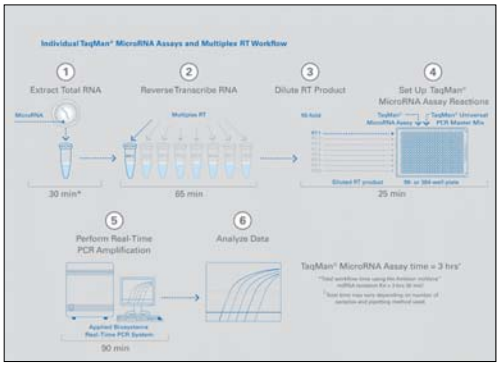


Figure 2. Total RNA was extracted from CRC cell lines using the MirVana small RNA Isolation kit (Ambion) to retian small RNA. A multiplex reverse transcription reaction was performed with 8 pools of miRNA primers. The synthesised cDNA was then profiled using TLDAs (Applied Biosystems) containing Taqman assays for 384 miRNA genes and including internal controls and normalisation genes. Gene expression was measured using real time RT-PCR and relative quantitation calculated by the delta Ct method.

Results

- Principle Component Analysis (PCA) was performed on normalised data. The greatest source of variability was observed for one of the batches (batch 2) of reverse transcriptase (RT) (Fig.3a).
- Effects of variation in Batch2 RT were removed by filtering the dataset for miRNAs showing expression in at least 50% of the cell lines leaving 177 miRNAs for further analysis. PCA was re-run on this subset (Fig.3b).
- In PC1 and PC2, the paired cell lines Vaco4A / Vaco4S, WIDR / HT29 and Colo201 / Colo206 showed the best clustering and SW480 / SW620 showed poor clustering (Fig. 3c).
- In PC1 and PC3, C84 and SKCO1 were identified as outliers and removed from further analyses (Fig. 3d).

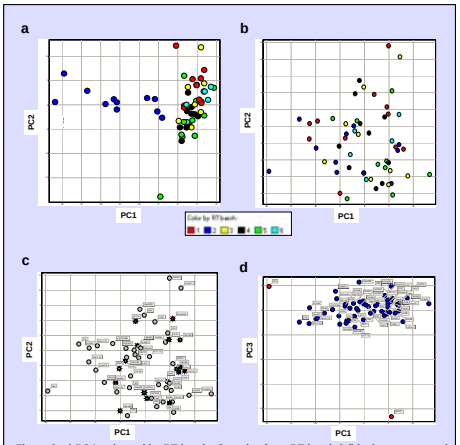


Figure 3: a) PCA coloured by RT batch. Samples from RT batch 2 (blue) are separated from the rest, showing batch effects; b) PCA re-run on filtered subset of 177 miRNAs showing removal of RT batch effect; c) PCA of subset showing how paired cell lines (coloured) cluster in PC1 vs PC2 and d) PCA on subset showing outlier cell lines SKCO1 & C84 in PC1 vs PC3.

- The potential for miRNA gene expression profiling to predict oncology drug sensitivity or resistance in the CRC cell line panel was tested.
- Four AZ oncology compounds AZC01, 02, 03 and 04 were selected and using pharmacology data sensitive and resistant CRC cell lines were determined for each compound. A two sample t-test was performed on normalised data assuming equal variance and using Storey's false discovery rate (FDR). For each compound, miRNAs within $P \leq 0.05$ and $FDR \leq 0.20$.
- A fold change in gene expression was used to determine the difference in expression between sensitive and resistant cell lines.

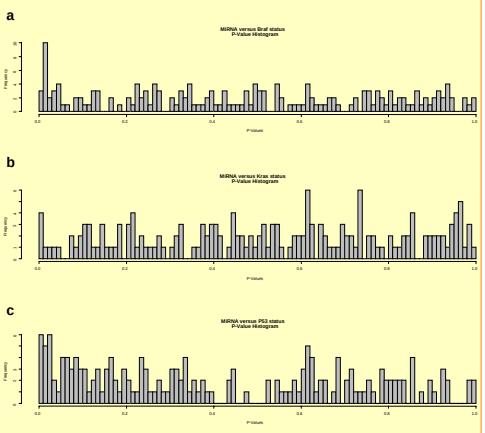
Compound AZC01			
miRNA	Pvalue	Mean sensitive-mean resistant (-delta Ct)	Fold change
miR-7	0.0004	2.6	6.2
miR-373	0.0005	-4.0	-15.9
miR-130a	0.0012	-3.6	-12.2
miR-372	0.0012	-6.3	-77.1
miR-192	0.0035	3.8	13.9
miR-371	0.0036	-3.4	-10.6
miR-520a	0.0037	-0.8	-1.7
miR-194	0.0040	3.7	12.9
miR-29c	0.0052	1.0	2.0
miR-206	0.0055	-1.1	-2.2
miR-335	0.0065	2.0	4.1
miR-512-5p	0.0096	-1.0	-2.0
miR-190	0.0114	2.2	4.5

Compound AZC02			
miRNA	Pvalue	Mean sensitive-mean resistant (-delta Ct)	Fold change
miR-7	0.0003	3.2	8.9
miR-373	0.0007	-4.0	-15.8
miR-130a	0.0009	-3.8	-13.5
miR-190	0.0009	2.8	6.8
miR-372	0.0015	-6.2	-74.6
miR-194	0.0035	3.8	13.5
miR-520a	0.0043	-0.8	-1.7
miR-371	0.0044	-3.4	-10.5
miR-196a	0.0056	1.9	3.8
miR-552	0.0059	3.5	11.4
miR-192	0.0062	3.6	12.1
miR-206	0.0066	-1.1	-2.2
miR-218	0.0096	-3.2	-9.5

Compound AZC03			
miRNA	Pvalue	Mean sensitive-mean resistant (-delta Ct)	Fold change
miR-548a	0.0002	1.4	2.6
miR-181d	0.0003	-2.4	-5.4
miR-412	0.0006	2.4	5.1
miR-181b	0.0034	-2.7	-6.4
miR-324-5p	0.0072	-1.5	-2.8
miR-376a	0.0073	0.2	1.2
miR-606	0.0073	1.4	2.6
miR-613	0.0073	0.7	1.6
miR-617	0.0073	0.8	1.7
miR-645	0.0073	0.7	1.7
miR-654	0.0073	0.9	1.8
miR-330	0.0079	-1.7	-3.2
miR-660	0.0104	1.1	2.2
miR-196b	0.0131	-2.6	-5.9

Compound AZC04			
miRNA	Pvalue	Mean sensitive-mean resistant (-delta Ct)	Fold change
miR-548a	0.0003	1.4	2.6
miR-181d	0.0004	-2.4	-5.3
miR-412	0.0007	2.3	5.0
miR-181b	0.0016	-2.6	-6.1
miR-330	0.0081	-1.6	-3.0
miR-376a	0.0083	0.2	1.2
miR-606	0.0083	1.4	2.6
miR-613	0.0083	0.7	1.6
miR-617	0.0083	0.8	1.7
miR-645	0.0083	0.7	1.7
miR-654	0.0083	0.9	1.8
miR-324-5p	0.0085	-1.5	-2.8
miR-660	0.0124	1.1	2.2

Figure 4. Table of microRNA within the parameters for the four selected compounds. 10/13 are the same for compounds, AZC01 and 02 (targeting MEK) and 13/13 for compounds AZD03 and 04 (targeting EGFR). The mean –ΔCt difference between the sensitive and resistant groups is shown, as is the fold change in expression of each microRNA. Positive values for mean sensitive – mean resistant, and fold change, indicate that the sensitive samples have higher expression than resistant samples.



Mutation	Global P-value
BRAF	0.139
KRAS	0.525 (multiple mutations)
P53	0.052

miRNA	p-Value	Fold Change Mutation / WT
miR-195	0.0005	2.0
miR-16	0.0017	1.7
miR-622	0.0029	8.6
miR-146b	0.0042	2.3
miR-15b	0.0069	1.8
miR-26a	0.0094	1.7
miR-501	0.0103	2.1
miR-30b	0.0106	1.6
miR-423	0.0120	1.6
miR-92	0.0156	1.7

miRNA	p-Value	Fold Change Mutation / WT
miR-17-3p	0.0181	1.9
miR-503	0.0222	2.0
miR-10b	0.0224	2.6
miR-650	0.0240	1.8
miR-223	0.0247	5.0
miR-34a	0.0268	-1.8
miR-146a	0.0281	5.6
miR-26b	0.0312	1.6
miR-19b	0.0314	1.5
miR-15a	0.0465	1.7

Figure 5. The linkage between mutation status for BRAF, KRAS and P53 and miRNA gene expression of colorectal cell lines was tested. Statistical analyses were performed and P-value histograms plotted for (a) BRAF: 47 cell lines; 37 WT & 10 mutation, (b) KRAS: 45 cell lines; 20 WT & 25 mutation and (c) P53: 44 cell lies; 10 WT and 34 mutation. The most significant linkage for BRAF and miRNA gene expression was demonstrated. Permutation testing revealed that the linkage between P53 status and miRNA gene expression was highly significant (p > 0.05). Tables show the miRNAs most significant gene expression change and relationship with p53 status.

Conclusions

1. It has been demonstrated by these studies that real time RT-PCR used to measure gene expression of miRNA with low density Taqman arrays (Applied Biosystems) is a robust and sensitive platform.
2. Preliminary analyses of sensitivity in colorectal cell lines have identified 13 significantly, differentially expressed miRNAs common to two oncology compounds that are EGFR inhibitors. Additionally, 10 miRNAs were found to be common and significantly,differentially expressed for two MEK inhibitors. Further statistical analysis combining the expression for multiple miRNA could identify gene signatures that predict sensitivity to compounds.
3. For this panel of CRC cell lines statistical significant changes in a subset of 177 miRNA gene expression consistent with P53 mutational status have been detected. Further data analyses are being performed to understand the relevance of those miRNAs showing the most significant changes and overlay biological findings.

References & Acknowledgements

Acknowledgements to the Cell Panel team for providing cell line materials for sample preparation. To Sara Davenport and Natalie Byrne for Pharmalogical data. To Christine Chresta and Mark Hickinson for advice.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005). microRNA expression profiles classify human cancers. Nature; 12: 900-909.