Reviewer's report

Title: Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer.

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Reviewer: Claus Lindbjerg Andersen

Reviewer's report:

Review of the manuscript entitled
“Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer”
By Greg M. Arndt et al.

Arndt et al. generates miRNA profiles from 45 tumor samples and four normal mucosa samples. All fresh frozen samples. Based on these profiles they identified 37 miRNAs deregulated between normal mucosa and cancer. They further specified which miRNAs were deregulated in early and late stage tumors. They also compared their array based measurements to measurements made using ABI tagman miRNA assays. Importantly, the authors also measured miRNA expression levels in a limited number of FFPE samples.

miRNA expression profiles were also generated from four cell lines. A minor fraction of the miRNAs deregulated in the clinical samples were also found to be deregulated in the cell lines. Next SW620 cells were stably transduced with miR143 and miR145 expression vectors. The authors observed that the reconstitution of miR143 expression lead to growth repression, while reconstitution of miR145 expression lead to augmented growth. Next they compared the predicted targets of the two miRs and found that no significant overlap existed between the pathways in which the predicted targets operate. They then searched previously published mRNA transcriptional profiles for the SW480 and SW620 cell lines and found that 55 of the 450 predicted miR145 targets were differentially expressed between the two. Based on a gene set enrichment analysis of the 55 genes they found that 4 pathways were significantly altered in the metastatic setting.

General comments:

The news value of the present manuscript is limited. Several miRNA profiling studies based on larger sample sets than the present have already been published. Similarly, several studies have already investigated the effects of miR143 and 145 reconstitution in in vitro models. As the authors state themselves the finding that miRNAs can be “reliably” measured in FFPE tissues has great perspectives. It would significantly strengthen the manuscript if FPPE tissues matched to all 49 fresh frozen samples were investigated. If the authors could show a significant overlap between the miRNAs found to be diff. expressed
based on fresh and FFPE tissue this would be great news. If the authors further collected clinical follow-up information for the tumor samples and could show that FFPE tissues could be used to identify prognostic miRNAs then this would greatly improve the news value of the manuscript.

- Major Compulsory Revisions

Comment 1: regarding “miR-145 has an oncogenic effect in metastatic CRC cells”

On page 11 the authors write: “Therefore miR-145 may be an activator of the transition to a more mesenchymal-like phenotype thus advancing tumor evolution, whereas miR-143 may contribute as a guardian of the epithelial-like state (both consistent with their opposing impacts on the metastatic cell model SW620). This was also supported by the observation that the ratio of miR-145 to miR-143 expression approximately doubled in late stage CRC.”

The authors state in Table 2 that miR145 is downregulated in all stages. Mir-145 is not in Table 4 with miRNAs differentially expressed between early and late stage. From figure 1 it is clear that nearly all cancer samples have very low levels of miR145. The authors have provided no evidence that up-regulation of miR145 occurs in late stage tumors. That the average ratio between miR145 and 143 (both expressed at very low levels in the cancer samples) differs between early and late stage does not at all imply that miR145 is up-regulated it could just as well be that mir143 is even further down-regulated in late stage samples..... However, considering the data provided in table 2 and figure 1 it more likely that observation reflects noise.

The observation that miR145 stimulates growth might very well be an artifact of the SW620 cell line. In particular when one considers that miR145 has been reported to repress growth in at least four other CRC cell lines SW480, DLD1, LS174T and HCT116.

Since miR145 up-regulation in late stage cancers is not observed and since the oncogenic function of miR145 is only observed in a single cell line - it is seems most likely that it is an cell line artifact with no basis in real life.

The authors should tone down the importance of the SW620/miR145 findings or provide data from clinical samples supporting the oncogenic function. It would also add significant strength to the manuscript if they could demonstrate the oncogenic function in at least one other cell line.

Comment 2: regarding: Figure 2. Correlation of miRNA expression comparing the mirVana Bioarray and ABI Taqman platforms. Linear regression was performed on 19 miRNAs that were measured on both platforms. Four different CRC samples were examined. Correlations ranged from 0.85 to 0.92.

2A) Figure 2 The sample IDs are different from what is listed in Table 1

2B) The IDs of the 19 miRNAs should be provided as well as the ABI product numbers
3B) Why are the comparison only shown for 19 miRNAs ??????? this seems strange considering that in figure 3 the authors have investigated 26 miRNAs in the same four samples using the type of ABI Taqman assays. The authors should show the data for all 26 miRNAs in Figure 2.

4B) Comparing expression levels of different transcripts by comparing the Ct values obtained with different real time assays (or array probes) is not so straightforward as is indicated.

Sure, for a single assay a sample with a low Ct value has a higher transcript level than a sample with a high Ct value. While this relationship is clear for a single assay, the same is certainly not guaranteed between independent assays (or probes).

To convince the reader that the qRT-PCR and array platforms produce similar results the authors should show that the two platforms find the same miRNAs differentially expressed when the same samples are investigated. They could select 5-10 miRNAs that the array platform indicated were differentially expressed e.g. miR-93, miR-183, and miR30a-3p which according to Table 2 were differentially expressed between normal and cancer and miR-31, miR-7, miR-125a which according to Table 4 were differentially expressed between early and late stage tumors)

- Minor Essential Revisions

Comment 3: regarding Profiling miRNA in clinical CRC samples

On Page 6 the authors write “Hierarchical clustering showed that many of these miRNAs were coordinately expressed, including the miR-143-145 and miR-17-92 clusters, which were consistently down- or up-regulated in CRC, respectively (Fig. 1).”

How were the miRNAs in the hierarchical cluster analysis selected? With other words Is it a supervised or unsupervised cluster analysis? That information is critical for the reader to able to interpret the figure.

Comment 4: regarding Profiling miRNA in CRC cell lines

On page 7 the authors write "To identify a CRC cell line for functional analysis of differentially expressed miRNAs, and to examine the relationship between miRNA expression in clinical samples and cell lines, we compared miRNA expression between normal colonic epithelial cells and four CRC cell line models (SW480, SW620, KM12C, KM12SM). From this analysis, 43 miRNAs were identified as either 2-fold up- or 2-fold down-regulated in at least one of the four CRC cell lines (Table 5). To validate the cell line microarray data, we performed Northern blot analyses on 22 of these miRNAs in these and an additional 4 cell lines (Fig. 4).

miRs 98, 221, 182, 27a,103,let7a, and 155 from figure 4, are not in Table 5
According to table 5 only 14 miRNAs were confirmed by Northern, while in the text it says 22.

How were the additional miRNAs in figure 4 selected for Northern?

Comment 5: Regarding the in vitro and in silico analysis of mi143 and 145:

5A) The authors should motivate why they, out of the 37 miRNAs in Table 2, chose miR143 and 145 for in vitro studies?

5B) On Page 6 the authors write “However, it was noted that late stage CRC samples (Stage IIIB and IV) exhibited twice the level of miR-145 expression compared to miR-143 (slope of 1.92).” Is the same the case for some of the early stage cases?

According to Table 4 miR143 and 145 were not differentially expressed between early and late stages.

According to figure 1 there is almost no miR-143 and 145 expressed in the CRC samples - could the observation be related to noise?

If the authors think the observation is important they should show the data so that the readers can evaluate the interpretation!!!

5C) On page 8 the authors write “The level of miR-145 approximated those observed in normal colonic epithelial tissue (data not shown).” The authors should describe how they did this comparison and show the data.

5D) The authors should motivate why they grow the cells with and without serum?

5E) Why is there so large a difference in the results obtained with the AS oligo on the vector control in figure 6C and 7B? What is the explanation for the growth suppression observed for the AS oligo on the vector control when cultured serum free???

The authors should comment on this.

5F) One page 8 the authors write “As seen in the previous experiment (Fig. 7C), over-expression of miR-145 in the presence of sense control RNA resulted in increased cell proliferation in serum, and more markedly in serum-free medium (Fig. 7B).”

This sentence is confusing and should be reformulated. Moreover, should not the reference to Figure 7C have been to Figure 6C?

Comment 6:

In the discussion (page 10) the authors write “Interestingly, the muscle specific miRNAs, miR-1 and miR-133a, were highly expressed in normal colon mucosa but were significantly down-regulated in CRC, indicating these miRNAs are not confined to muscle [32].”
Apparently the authors believe that the reduced levels of miR-1 and 133a observed in the tumor samples indicate that the expression of the expression of the two miRNAs are not confined to muscle cells. An alternative explanation could be that there are differences in the tissue composition (muscle cells) of the investigated cancer and normal mucosa specimens. The authors should comment on this!

Alternatively, they could use in situ hybridization to demonstrate that miR-1 and 133a are predominantly expressed by epithelial and not muscle cells in normal mucosa samples.

Comment 7: regarding the description of the Clinical Samples

On page 14 the authors write “In total, 49 fresh-frozen human tissue samples were obtained from Genomics Collaborative Inc. (Cambridge MA) or Clinomics Bioscience, Inc (Pittsfield, MA), including 4 normal colon, 4 Stage I, 19 Stage II, 20 Stage III and 2 Stage IV samples (Table 1). In addition, we obtained 8 matched formalin fixed paraffin embedded (FFPE) samples (3 Stage II, 4 Stage III and 1 Stage IV).”

6A) Table 1 contains 64 clinical samples and not 49. Even adding the 8 FFPE samples does not give 64. What is the explanation?

6B) The sample IDs in the table are not identical to those used in the figures, why is that?

6C) It is well known that two major molecular subtypes of CRC exists the one being mismatch repair deficient (MSI) and the other proficient (MSS). Is the MSI/MSS status known for the investigated samples? Several papers have shown that miRNAs are differentially expressed between the MSS/MSI subtypes potentially this could confound the findings in the present study.

6D) Thorough information about the four normal colon specimens is needed. As these specimens are the reference material for the entire study, at least this must include information on how the specimens were collected, under which circumstances (surgery, bowel endoscopy, rectoscopy), previous and/or concurrent diseases, are the specimens from non-cancer areas of cancer patients, etc.

Comment 8: regarding Figure 1. Two-way hierarchical clustering of CRC and normal colorectal tissue using 37 differentially expressed miRNAs.

The sample IDs in figure 1 are not consistent with Table 1 (listing the clinical characteristics of the clinical samples). This makes it nearly impossible for the reader to evaluate and interpret the figure.

Comment 9: regarding Figure 3. miRNA expression in fresh frozen versus formalin-fixed paraffin embedded CRC samples. (A) Taqman miRNA expression assays were performed on 26 miRNAs from 4 matched fresh frozen and FFPE samples. (B) Comparison of expression of 18 miRNAs from 2 matched fresh frozen and FFPE samples using the mirVana Bioarray assay.
9A) The sample IDs in figure 3 are different from those listed in Table 1. This should be changed.

9B) According to the description of the clinical samples (on page 14) eight FFPE samples were selected for analysis. However, according to legend to figure 3 only 6 FFPE samples were investigated. Why is that?
Why were not all eight samples investigated by both the mirVana Bioarray assay and the Taqman miRNA expression assays?

9C) It appears that panel B with the results from the mirVana Bioarray assay is missing in figure 3.

9D) The IDs of the 26 miRNAs should be provided as well as the ABI product numbers
- Discretionary Revisions

Comment 10
On page 4 the authors write “Several miRNAs have been identified as differentially expressed between normal and tumor tissues or cancer cell lines [20]. In CRC, there have been limited studies examining the expression patterns of miRNAs [21-25].”

The authors should acknowledge the existence of larger miRNA transcription profiling studies in CRC than those cited.

For example:
MicroRNA Expression Profiles Associated With Prognosis and Therapeutic Outcome in Colon Adenocarcinoma
Aaron J. Schetter; Suet Yi Leung; Jane J. Sohn; et al.

Diagnostic and prognostic microRNAs in stage II colon cancer.
Schepeler T, Reinert JT, Ostenfeld MS, Christensen LL, Silahtaroglu AN, Dyrrskjøt L, Wiuf C, Sørensen FJ, Kruhøffer M, Laurberg S, Kauppinen S, Ørntoft TF, Andersen CL.

In both these studies more CRC samples were investigated than in the present study.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable
**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

'I declare that I have no competing interests'