Author’s response to reviews

Title: Expression of miR-21 and its targets (PTEN, PDCD4, TM1) in flat epithelial atypia of the breast in relation to ductal carcinoma in situ and invasive carcinoma

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Author’s response to reviews: see over
Reviewer 1
This manuscript compares the expression of miR-21, and three previously established targets of miR-21, in normal breast, flat epithelial atypia (FEA), ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC).

Discretionary Revisions
1. On page 5, the statement “Zhu et al proved” is an overstatement
We have adapted this sentence in the introduction

Minor Essential Revisions
1. In the abstract, it is stated “…15 cases with simultaneous presence of normal breast tissue, FEA, DCIS….” Does this mean all 3 were present in every case? If so, insert an “and” before DCIS.
All three components were present in 12 of the 15 cases, we have adapted the sentence to FEA and/or DCIS.

2. On page 4 “in about 30%” should read “in about 30% of patients”
We have changed this according to the suggestion of the reviewer.

3. On page 4, what percentage of FEAs show the genetic alterations mentioned (i.e. LOH on 3p, 9q, …)?
We have added the range of percentages of LOH observed in FEA to the introduction.

4. On page 4, second to last line, “MiRNA-21” should be “MiR-21”
We have adapted this mistake.

5. On page 5 “Lorio at al” should be “Iorio et al”
We have corrected the reference.

6. On page 6, “in the introduction” should read “in the Introduction”
This has been adapted according to the suggestion of the reviewer.

7. On page 6, it sounds like only one institution followed Code of Good Conduct and had an IRB. The authors need to explicitly name both Institutions here.
We have now included statements for both institutions separately.

8. On page 7, “phosphatate” should read “phosphatase”
This has been adapted.

Major Compulsory Revisions
1. On page 8, “all appropriate negative controls” need to be stated explicitly
We adapted the methods section and explicitly stated which controls have been used.

2. The results in Figure 1 are reasonably convincing for the 5 patients shown. However, are the authors sure that all fields are 200x magnification?
We have checked the magnification and adapted the figures that were not correct. We apologize for this mistake.
If so, the normal tissue should include large ducts, as shown for FEA, instead of just TDLUs.

FEA is a disease of the lobuli rather than of the ducts and for this reason we show lobuli for both normal breast tissue and for FEA in figure 1. In general FEA shows dilated acini, which explains the difference in size of normal and FEA lobuli in the used picture.

Also, the investigators need to perform microRNA ISH on these same blocks with a control microRNA whose levels do not change, or decrease from normal to DCIS. Actin mRNA is very different from microRNAs in size, and not a convincing indicator that microRNAs in general were well preserved and retained during the entire process. We agree with the reviewer that Actin is not a good control for preservation of miRNAs in tissue. The reason that we used Actin as a quality control was because this gene is highly expressed in all types of cells, and this staining does give us good information about the consistency of the fixation within a tissue section. We used this staining merely to determine if the tissue fixation was homogenous. We have more clearly indicated this specific point now also in the methods section (under Specimen collection). Selection of a miRNA that does not change from normal to FEA and DCIS is difficult since the number of miRNA ISH studies are very limited, thus far only one study has been reported for breast tissue.

3. On page 9 at the bottom, it is stated that “In twelve samples, all three components were present in the tissue section and a marked progressive increase in staining intensity was observed from normal to FEA and further to DCIS in five cases.” This sentence needs to be broken up into two sentences. We have adapted this sentence according to the suggestion of the reviewer.

But more importantly, only a minority of the small sample size displayed this progression. The authors barely address this. Does this mean that only some FEA are preneoplastic?? Also, the conclusion (page 14) that “the presence of miR-21 in FEA suggests that FEA has to be regarded as a premalignant lesion” is not really warranted. What about the 8 cases of FEA with no miR-21 expression?

We agree with the reviewer that we put to much emphasis on the 5 cases showing the increase in miR-21 expression. We have adapted both the results and discussion section and now also describe more in detail the staining pattern in the other 10 FEA cases. Moreover, we have adapted our conclusions with respect to the pre-malignant character of FEA.

4. The author’s state that PTEN is positive and variable in all tissues (although the series shown in Fig 3 appears to have less staining in DCIS and IDC). Based on this, the authors state on p12 “…it seems unlikely that PTEN represents a true target gene for miR-21 in breast cancer” The apparent lack of an inverse correlation between miR21 and PTEN does not indicate that PTEN is not a target. Many other factors could explain this, and in some cases of breast cancer, PTEN may be dominantly repressed by miR-21. The investigators should at least try to overexpress miR-21 in a breast cancer cell line and assay PTEN protein levels.

We agree with the reviewer that we can not conclude that PTEN is not a target, this might still be true in some cases of breast cancer or in other cancer types. We have
adapted the text parts and more carefully draw our conclusions. We added supplementary tables, containing the results of a detailed, case-by-case analysis, to further illustrate the lack of a real association in our study.

5. Higher magnification of PDCD4 is required to allow the readers to observe nuclear vs cytoplasmic localization for themselves. We have improved the quality of the picture and show a higher magnification to illustrate more clearly the difference between cytoplasmic and nuclear staining of PDCD4.

The statement that “it is highly unlikely that PDCD4 is regulated by miR-21” does not consider the possibility that a protein involved in nuclear vs cytoplasmic localization of PDCD4 is a target of miR-21. But see my comment above concerning conclusions made from the lack of an inverse correlation.
We have adapted the discussion and included the suggestion as mentioned by the reviewer.

Again, in Figure 3, it would be useful to compare large ducts in normal to the large ducts in FEA.
The photograph indeed shows only a lobule. As stated in the introduction, FEA is actually regarded as a disease of the lobuli, rather than the ducts. For this reason the photomicrograph is an appropriate illustration of our message. See related comment above.

6. In summary, the identification of miR-21 in some specimens of FEA suggests that, in some cases, FEA may indeed be preneoplastic. However, the 8 samples of FEA that were negative for miR-21 do not support this conclusion.
We have adapted the summary and also report the lack of miR-21 in FEA components for which the DCIS components were positive in the summary.

Further, although miR-21 is upregulated in numerous types of cancer, I am not aware of any study that suggests that its expression alone in a normal mammary epithelial cell is sufficient to cause transformation.
We agree that there is no proof that miR-21 induces malignant transformation and rephrased this in summary and discussion. However, some papers do report a function of miR-21 in invasive growth, proliferation and metastasis in cell lines supporting an oncogenic role for miR-21.

Reviewer 2
In this article the authors examine the expression of miRNA-21 and some of its molecular targets in a small series of clinical breast samples including regions of normal breast epithelium, flat epithelial atypia (FEA), DCIS and invasive ductal cancer; the aim is both observational but also to add to the data concerning whether FEA shares features with DCIS or invasive cancer and may, therefore, be a pre-malignant lesion. If FEA were shown to be a pre-malignant condition this might have important consequences for the clinical management of breast cancer – consequently the study is of considerable interest and is certainly appropriate for publication in BMC Cancer. In addition, the assessment of expression of miRNA-21 and its potential targets – thereby
establishing which potential targets are likely to be actual targets in the clinical setting – is of potential interest.

In fact, the data in the article do not support any firm conclusions concerning the premalignant nature of FEA or concerning miRNA-21 targets. Despite this, I believe that the article contains plenty of novel and interesting observational data that is clearly presented; I recommend that the article should be accepted subject to changes to the text (and even the title) – as detailed below. Many of these are to improve statistical over- or misinterpretations. In addition, it may be possible to include some further simple explanations and analyses.

In the terms of this journal, I would class all of these points as major compulsory revisions.

1) Title: In the body of the article the authors are clear about the fact that the increase in miRNA-21 expression they see in FEA is only a statistical trend – however this is not clear from the title. A title merely stating the observational aim of the study might be better.
   We have adapted the title.

2) Abstract: Conclusions. The authors state that the “TM1 staining patterns showed an inverse correlation with miRNA-21”. Is that actually correct? Or is it merely the case that TM1 correlated negatively with progression, while miRNA-21 correlated positively with progression – this does not necessarily mean that they negatively correlate with each other. Have the author actually tested this negative correlation?
   We agree with the reviewer that the fact that we do not see a difference does not indicate that they are similar. We have adapted this section of the manuscript and more carefully draw our conclusions.

   In particular, TM1 staining in normal and FEA look very similar (Table 3) – while it is one of the authors’ main assertions (the title) that miRNA-21 differs in these two tissues. Also, TM1 staining in FEA and DCIS look rather different – while the authors again assert that miRNA-21 expression is similar in FEA and DCIS (final conclusion section). These observations point towards TM1 having no clear relationship with miRNA-21. This point also applies to text concerning miRNA-21 and TM1 towards the end of the discussion.
   We agree with this reviewer that although we do see an inversed staining pattern, this does not indicate that there is an inverse correlation. Moreover, the inversed staining pattern seems to be specifically true for normal as compared to DCIS and IDC and not for FEA. We have adapted this part of the text.

3) Methods: It surprised me that the authors felt it necessary to exclude blocks with poor actin ISH signals from the miRNA ISH – an exclusion not always done (eg Sempere et al Cancer Research 2007). MiRNA are generally felt to be more resistant to degradation that mRNAs – therefore I suspect that miRNA ISH may work perfectly well on these blocks. Do the authors have data to support their exclusion?
This point was also addressed by reviewer 1 (major compulsory point 2) and had been adapted in the text. In general, we observed that the signal and the area of the tissue section showing a good signal for B-actin is more extensive than the area showing a good signal for miR-21. In a few cases the staining appears to be more prominent for miR-21 as compared to B-actin. We have now also included this observation in the results section of the revised manuscript.

Or alternatively, could the authors try the ISH on these blocks, which could add to the size of the cohort and thereby may allow statistical trends to reach significance? The most important reason to perform the Actin staining in this study was to determine if the fixation and quality of the blocks are homogeneous. This is very important for our study since we want to compare different components within one tissue block and we have seen large variation in the Actin intensity throughout a single tissue block. This might lead to false negative scoring of normal, FEA or DCIS components. See also point 2 raised by reviewer 1.

4) Methods: for miRNA-21 ISH could the authors be more specific about the “appropriate negative controls” that were included? We have added the controls used for the staining in the methods section.

5) Methods: IHC staining. Have the authors done any validation to show that these antibodies detect the antigens they are meant to? Can the authors cite any other published use of these antibodies for IHC – or have they performed Westerns to show their specificity? We did not perform Western blotting for these antibodies, since these antibodies have been applied in many other studies previously and shown to yield a specific staining pattern. We compared our staining pattern observed in control tissues to previously published data. We have included references for the different antibodies and controls used for the IHC.

6) Results: miRNA-21 ISH. The author stated that there is a “marked progressive increase in staining intensity” from “normal to FEA and further to DCIS in five cases”. In fact, in Table 2, they show that have scored FEA and DCIS as the same (“+”) in 4 of these cases – what do they mean by a progressive increase across these tissues? We agree with the reviewer that this statement is not correct. We have adapted the results section and clearly state that there is an increase from normal to both FEA and DCIS and removed the progressive increase. We also added this to the figure legend, to prevent confusion.

In fact, the data support a trend between normal and FEA, and a difference between normal and DCIS – but not a progressive increase.
We agree with the reviewer that we put to much emphasis on the five cases that do show a trend and adapted this part of the paper. However, we do see an obvious increase in staining intensity in 6 out of 15 cases comparing the normal to the FEA component. The same point has been addressed by reviewer 1.
7) Results: miRNA-21 ISH last sentence. The fact that there was no statistically significant difference between miRNA-21 expression between FEA and DCIS does not indicate a similar pattern of staining. It merely shows the lack of a statistically significant difference. It is possible to do a separate statistical test to assess whether the staining is similar – this would support the statement.
This point is related to the previous remark and we accordingly adapted the text in results and discussion of the revised manuscript. We more carefully draw our conclusions with respect to the similarities between FEA and DCIS throughout the manuscript.

8) Results: PTEN, TM1 and PDCD4 staining. Throughout the section the authors have analysed expression with respect to tissue type. Could the authors attempt to correlate marker expression with the matched miRNA-21 expression data – thereby directly testing the correlation (see also point 2)?
We have now included a separate paragraph to compare the miR-21 staining directly to the target gene staining patterns case by case for each component. These data have now also been added as supplementary files.

9) Discussion. “We observed no obvious difference for expression of miRNA-21 between FEA and DCIS supporting a relation between FEA and DCIS” – as point 7 – a lack of a significant difference does not necessarily show they are similar.
This is the same point as raised by the previous reviewer and we have adapted the manuscript according to the suggestions of both reviewers.

10) Discussion. The discussion of each marker as a potential miRNA-21 target would be greatly aided by the analyses suggested in point 8.

The reason for the extensive correlation of the staining pattern for the target genes with the miR-21 pattern is that we do believe that it is important to validate cell line results in primary tissue samples. We do realize that in this way we merely can observe an inverted relation and can not confirm a direct targeting. Moreover, as suggested in point 8 we now also include a supplementary table showing the detailed results per case.

11) Conclusions. Points 2 and 7 apply to this section also.
We have adapted the text according to the suggestions of the reviewer.

Reviewer 3
The authors in their article “Elevated expression of miR-21 in flat epithelial atypia of the breast” have presented evidence of progressively increased expression of miR-21 in FEA to DCIS to IDC and claimed that based on the data, FEA should be considered as premalignant lesion. The actual significance of FEA in breast cancer is controversial and only increased miR-21 expression may not be sufficient for this consideration. MiR-21 upregulation in cancer, particularly in breast cancer is well established and the targets investigated by the authors are also validated. Based on the literature the authors examined the expression of three targets and found two of them to be reciprocally regulated by miR-21.
Understanding the early events in breast tumorigenesis is becoming increasingly important. Deregulation of microRNAs in cancer is widely studied in the last few years. Therefore, identification of deregulated miRs at early stages of breast cancer is important and can lead to deciphering the underlying mechanism that leads to carcinogenesis.

Major compulsory revision
1) Since Real-time PCR for miR-21 is well standardized, real-time data will allow more quantitative comparison of miR-21 expression between the different stages of carcinoma.

We agree with the reviewer that qRT-PCR is a much more quantitative approach, however, since all tissue blocks contain a mixture of different cell types it is not possible to correlate expression levels to FEA and DCIS. This can only be achieved by coupling the procedure to laser microdissection of specific compartments followed by qRT-PCR, this is technical very difficult for paraffin embedded tissue and beyond the scope of the study.

2) PDCD4 staining is not very impressive and the claim that the localization switches from nuclear to cytoplasmic compartment is not obvious. A higher magnification of the picture will help the future readers to appreciate this relocalization of PDCD4.

This point was also raised by the previous reviewer and we have according to their suggestions improved the pictures and included a higher magnification.

Minor essential revision
1) In page 5, TM1 stands for Tropomyosin 1.

We have corrected this mistake.

2) TDLU: not abbreviated.

We have replaced this term by normal breast tissue.

3) The catalog number provided in Table 1 for TM1 antibody corresponds to a different protein.

We corrected the catalogue number and apologize for the typo.

Discretionary Revisions
The authors may want to provide concurrent miR-ISH and target-IHC in the same tissue section, to further validate their observation.

Unfortunately, the staining for the three target genes has been performed at a later stage as compared to the miR-21 staining and several sections have been cut in that time for different purposes. We tried to find the same histological area back in the miR-21 staining, but the pattern was too different from the pattern in the IHC staining and we were unable to add miR-21 pictures for the same tissue areas in this case.