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Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers

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1st Editorial Decision 09 November 2010

Thank you for the submission of your manuscript "Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers" to EMBO Molecular Medicine and please accept my apologies for the delay.

We have now received two out of three referee reports on it and given that the review process so far has been lengthy, we would prefer to make a decision now. We will send you the third report as soon as it becomes available.

Although the reviewers find the study of potential interest, especially reviewer #3 raises a number of significant concerns about the lack of functional data and several technical issues. However, we would be willing to consider a revised manuscript with the understanding that the referee concerns must be convincingly addressed.

In particular, reviewer #3 feels that additional functional characterization would significantly strengthen the study and suggests several experiments to achieve that. The reports are explicit and I will not repeat the suggestions here. However, we feel of particular note is the investigation of drug responses after miRNA overexpression and a more detailed analysis and description of the tumour samples.

We realize that addressing all of the referees' criticisms might require a lot of additional time and effort and be technically challenging. Should you decide to embark in such a revision, revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless discussed otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1 Remarks on Novelty/Model system:

This is a very innovative study which addresses a very important question in breast cancer. The results from this study likely help explain why BRCA1 expression tends to be low in triple negative breast tumors.

Referee #1:

The statistical analysis needs more detail. It would be nice to see more correlations between miR146a/miR146a-5p expression and clinical variables, such as, tumor grade, stage, etc.
The discussion on miR-146 and inflammation is interesting. However, it would be more interesting for the authors to look at some inflammatory biomarkers in breast tumors and correlate them with miR-146a.
Discussion on ovarian cancer is a little bit excessive. It should be shortened.

Referee #3 Remarks on Novelty/Model system:

Insufficient mechanism / clinical implications

Referee #3 Summary and context:

In this paper the group investigate the role of miRNA as a novel mechanism controlling BRCA1 expression. BRCA1 expression is reduced in up to 30% of sporadic breast cancers and this low level of expression cannot be completely explained by currently established mechanisms such as somatic mutations, promoter methylation, LOH etc.

In this paper bioinformatics tools were used to predict miRNAs that would bind the BRCA1 transcript 3'UTR. Of the 14 possible binding miRNAs generated, those implicated in breast cancer were taken forward for further investigation (miR-9, miR-17-5p, miR-146a and miR-146b-5p).

General comment

This is an interesting paper describing a potentially novel mechanism for controlling BRCA1 expression. However, the major limitation of this paper is the lack of functional experimental data showing that a reduction in BRCA1 expression mediated by these miRNAs leads to physiologically relevant consequences such as sensitisation to DNA damaging agents or PARP inhibitors. As it stands we feel the paper is only suitable for publication in a specialist journal but the addition of functional data would strengthen the arguments made in the manuscript.

Detailed comments

1. For publication in a journal such as EMM the manuscript needs to be significantly improved by the addition of more functional data. For example, the effect of the miRNA on homologous recombination should be further studied using additional assays such as gamma H2AX and RAD51 foci formation. In addition, the loss of BRCA1 via these miRNAs should render cells sensitive to DNA damaging agents such as mitomycin C and gamma irradiation and also to PARP inhibitors. Drug dose response studies are required. These sensitisation effects could then be reversed using the anti-miRNAs to prove the pivotal role of the miRNAs in this effect.
2. Figure 1. The western blot in figure 1D needs to show individual miRNA transfections showing

knockdown of BRCA1 with either miRNA, not just the combination as shown.
3. RISC IP or a similar technique needs to be used to prove that BRCA1 binds to these miRNAs.
4. Figure 3: The western blot in figures 3A and C would benefit from the addition of individual miRNA or anti-MiRNA transfections showing knockdown of BRCA1 with either miRNA or anti-miRNA, not just the combination as shown.
5. Please provide a reference for the statement at the top of page 10 "BRCA1 has been repeatedly shown to inhibit cellular proliferation when overexpressed in different cell types".
6. Figure 5: Relationship between BRCA1 expression and miRNA expression is limited by the fact that the table provided does not show whether, for example, the tumours that are negative for BRCA1 are the same ones that have a high level of the miRNA. In the western blot example it would be useful to know which tumour this is (from supplementary table 3) and to see the blots for all the 35 tumours analysed in this way. Why were the rest of the tumours not suitable for analysis and only one example shown? How were BRCA1 presence and absence defined? How were intermediate cases graded? There is no statistical significance test performed on the data in figure 5B and no attempt at formal correlation between BRCA1 loss and miRNA expression, which limits interpretation.

7. Figure 6: Relationship between miRNA - 146a/b-5p expression and hormonal status: The comparison of expression median in triple negative with all tumours is not relevant. It would be more relevant to show the comparison of triple negative to non-triple negative tumours. It is not clear what the association with hormonal status adds to the overall message of the paper.

8. Page 3 - line 2: Please provide a reference for the first statement of the introduction.
Page 3 - line 5: Please reference a reference for "they account for no more than 1-2% of all breast cancer cases".

1st Revision - Authors' Response 02 February 2011

Referee #1 Remarks on Novelty/Model system:

This is a very innovative study which addresses a very important question in breast cancer. The results from this study is likely to help explain why BRCA1 expression tends to be low in triple negative breast tumors.

Referee #1:

The statistical analysis needs more detail. It would be nice to see more correlations between miR146a/miR146a-5p expression and clinical variables, such as, tumor grade, stage, etc.

We have reinforced the statistical analysis of the results obtained in the series of 167 breast tumours by correlating miR-146a/miR-146b-5p expression levels not only with receptor status and HER2 amplification, but also with tumour grade, tumour stage, presence of metastasis and menopausal status. The paragraph dealing with this issue in the result section has been amended and now reads as follow:

“We found that miR-146a/miR-146b-5p expression levels are significantly higher in triple negative versus non triple negative, in ER-PR- versus ER+ and/or PR+, and in SBR grade III versus grade II breast tumours (Fig. 5). miR-146a/miR-146b-5p expression levels were not found to be associated with ERBB2 amplification, pTNM stage, and metastasis or menopausal status (data not shown). These results are consistent with the fact that breast tumours developed by *BRCA1* mutation carriers commonly lack ER and PR expression, do not over-express ERBB2 and are of a higher grade than those found in controls.”

Figure 5 has been modified accordingly.

The discussion on miR-146 and inflammation is interesting. However, it would be more interesting for the authors to look at some inflammatory biomarkers in breast tumors and correlate them with miR-146a.

We have looked at two inflammatory biomarkers, TRAF6 and IRAK1, by Western blot analysis in HeLa cells and found that, as for BRCA1, IRAK1 levels were drastically reduced in cells transfected by miR-146a or miR-146b-5p precursors, while TRAF6 amounts were unchanged. We then proceeded to measure the amount of IRAK1 in parallel to BRCA1 in our set of 35 breast tumours for which we had tumour material (results shown in Figure S5 of Supporting Information), and found in IRAK1 negative and IRAK1 positive tumours a roughly equal distribution of tumours with miR-146 level < to median and with miR-146 level > to median. We have amended the manuscript as following:

First, in the "Repression of *BRCA1* expression by miR-146a and miR-146b-5p" section:

"To determine whether miR-146a or miR-146b-5p affected endogenous *BRCA1* expression, we compared the level of the BRCA1 protein in HeLa cells after transfection with miR-146a or miR-146b-5p synthetic precursors, or with a negative control precursor. We first showed by Northern blot analysis that miR-146a and miR-146b-5p could be detected in transfected cells only (Fig S1A of Supporting Information). The expression of these miRNAs individually or in combination led to a drastic reduction in the amount of IRAK1, a known target of miR-146a and miR-146b-5p (Taganov et al, 2006; Perry et al, 2008), and in the amount of BRCA1 protein (Fig 1D), demonstrating that miR-146a and miR-146b-5p are effective on the endogenous *BRCA1* gene."

Second, in the "miR-146a/b-5p expression in breast tumours" section:

"We observe a statistically significant inverse correlation between BRCA1 and miR-146 (P= 0.05), but not between IRAK1 and miR-146, which suggests that *IRAK1* gene regulation is probably complex and exerted by multiple actors upon different layers."

Discussion on ovarian cancer is a little bit excessive. It should be shortened.

The discussion on ovarian cancer has been shortened by half the original length. It now reads as below:

"Given the involvement of *BRCA1* in ovarian cancer susceptibility and possibly in sporadic ovarian cancer (Weberpals et al, 2008), it is interesting to note that miR-146b-5p has been found to be up-regulated in ovarian cancer tissues and cell lines (Dahiya et al, 2008), especially in stage III ovarian cancers (Eitan et al, 2009) and in the ovarian serous carcinoma sub-type (Wyman et al, 2009). As ovarian tumours developed by *BRCA1* mutation carriers are mostly stage III serous carcinoma (Lakhani et al, 2004), these results raise the possibility that miR-146b-5p and possibly miR-146a could also be involved in *BRCA1* down-regulation in sporadic ovarian cancer."

Referee #3 Remarks on Novelty/Model system:

Insufficient mechanism / clinical implications

Referee #3 Summary and context

In this paper the group investigate the role of miRNA as a novel mechanism controlling BRCA1 expression. BRCA1 expression is reduced in up to 30% of sporadic breast cancers and this low level of expression cannot be completely explained by currently established mechanisms such as somatic mutations, promoter methylation, LOH etc.

In this paper bioinformatics tools were used to predict miRNAs that would bind the BRCA1 transcript 3'UTR. Of the 14 possible binding miRNAs generated, those implicated in breast cancer were taken forward for further investigation (miR-9, miR-17-5p, miR-146a and miR-146b-5p).

General comment

This is an interesting paper describing a potentially novel mechanism for controlling BRCA1 expression. However, the major limitation of this paper is the lack of functional experimental data showing that a reduction in BRCA1 expression mediated by these miRNAs leads to physiologically relevant consequences such as sensitisation to DNA damaging agents or PARP inhibitors. As it stands we feel the paper is only suitable for publication in a specialist journal but the addition of functional data would strengthen the arguments made in the manuscript.

Detailed comments

1. For publication in a journal such as EMM the manuscript needs to be significantly improved by the addition of more functional data. For example, the effect of the miRNA on homologous recombination should be further studied using additional assays such as gamma H2AX and RAD51 foci formation. In addition, the loss of BRCA1 via these miRNAs should render cells sensitive to DNA damaging agents such as mitomycin C and gamma irradiation and also to PARP inhibitors. Drug dose response studies are required. These sensitisation effects could then be reversed using the anti-miRNAs to prove the pivotal role of the miRNAs in this effect.

The aim of our work was to identify new mechanisms leading to *BRCA1* down-regulation in breast tumours. Indeed, while numerous studies have shown in different contexts and using various assays and read-out systems how detrimental for genetic stability, and particularly for homologous recombination (HR), is the loss of expression of *BRCA1*, we still don't understand how this loss of expression is achieved in sporadic tumours.
Having shown that miR-146a and miR-146b-5p expression results in reduced BRCA1 levels, that inhibition of these miRs leads to an increase of the amount of BRCA1, and that miR-146 levels in breast tumours inversely correlate with BRCA1 levels or triple-negativity, we believe that we have convincingly unravelled a new mechanism leading to the loss of expression of *BRCA1*.
We have checked that this reduction in BRCA1 levels had the expected consequences on proliferation and HR. HR is a process by which exchange of genetic material occurs between two DNA strands, resulting in genetic recombination. Several assays can be used to monitor the HR mechanism : some of them are indirect and reflect the assembly of HR protein complexes, like RAD51, H2AX or 53BP1 foci. Indirect assays may be inaccurate, as foci can assemble even if HR is impaired (in the presence of dominant negative mutant RAD51 molecules for example) and therefore, it is important to confirm that they reflect HR efficiency by performing other experiments such as sensitisation to DNA damaging agents. Others, such as the sophisticated assay we have used in our study, directly measure genetic material exchange. As they truly reflect HR efficiency, it is not necessary to confirm them by additional experiments. Moreover, the most appropriate foci to study regarding BRCA1 involvement in HR would be 53BP1 foci rather than H2AX and RAD51 foci as suggested, given the interplay between BRCA1 and 53BP1.
Anyway, it does not appear useful in the context of this work to study more deeply the consequences of the loss of BRCA1 as this has been done extensively by others, and as there is absolutely no reason to suspect that the consequences of the down-regulation of BRCA1 through microRNAs should be any different from those of the down-regulation of BRCA1 through other mechanisms.

2. Figure 1. The western blot in figure 1D needs to show individual miRNA transfections showing knockdown of BRCA1 with either miRNA, not just the combination as shown.

The western blot in Figure 1D now shows knockdown of BRCA1 through transfections with either miR, not just the combination as shown in the previous version.

3. RISC IP or a similar technique needs to be used to prove that BRCA1 binds to these miRNAs.

To identify miRNAs targeting the *BRCA1* 3'-UTR, we performed a computational search using three different algorithms: MicroInspector, miRanda, and TargetScan. The *BRCA1* 3'-UTR was then examined with a fourth algorithm, RNA22, for potential binding sites for all the miRNAs predicted by the three previous algorithms. To limit the number of predictions, only the miRs predicted to bind their target with a folding energy below –25 Kcal/mol were considered for analyses. Furthermore, to reduce the number of false positives, only the miRs predicted by at least two algorithms were further considered, which was the case of miR146a (predicted by three algorithms) and miR-146b-5p (predicted by two algorithms). In a second step, in order to prove that miR146a and miR146b-5p do bind to the 3'-UTR of *BRCA1*, we have used the most acknowledged approach, i.e. luciferase assays. We inserted the 3′-UTR of *BRCA1* into a reporter plasmid at the 3′ end of the luciferase open reading frame and observed that miR-146a or miR146b-5p expression reduced luciferase activity. We then mutated two nucleotides within the seed region of the miR-146a- or miR-146b-5p target site in the 3′-UTR of *BRCA1* and observed that miR-146a or miR146b-5p expression no longer reduced luciferase activity. We concluded from these experiments that miR146a and miR146b-5p inhibit *BRCA1* expression by directly interacting with the *BRCA1* 3′-UTR.

Having shown this, we do not think that it is necessary for the purpose of our analysis to gain further insight into the mechanisms of action of miR-146a/miR-146b-5p. Indeed, most publications that describe a new microRNA-mediated gene regulation don't go beyond what we have shown.

4. Figure 3: The western blot in figures 3A and C would benefit from the addition of individual miRNA or anti-MiRNA transfections showing knockdown of BRCA1 with either miRNA or anti-miRNA, not just the combination as shown.

The western blot in Figures 3A and 3C now shows results obtained by transfecting either miR-146a or miR-146b-5p, or either anti-miR, not just the combination of both as shown in the previous version.

5. Please provide a reference for the statement at the top of page 10 "BRCA1 has been repeatedly shown to inhibit cellular proliferation when overexpressed in different cell types".

References have been added for the statement at the top of page 10 "BRCA1 has been repeatedly shown to inhibit cellular proliferation when overexpressed in different cell types" (Holt et al, Nature Genetics, 1996; Abbott et al, J Biol Chem, 1999; Aprelikova et al, PNAS, 1999).

6. Figure 5: Relationship between BRCA1 expression and miRNA expression is limited by the fact that the table provided does not show whether, for example, the tumours that are negative for BRCA1 are the same ones that have a high level of the miRNA.

The table in Figure 5B (now Table 1) has been redesigned for clarity. It is now easier to figure out that among the 15 breast tumours negative for BRCA1, 4 have a miR-146 level < median level, and

11 have a miR-146 level > median level, and that among the 20 breast tumours positive for BRCA1, 12 have a miR-146 level < median level, and 8 have a miR-146 level > median level.

In the western blot example it would be useful to know which tumour this is (from supplementary table 3) and to see the blots for all the 35 tumours analysed in this way. Why were the rest of the tumours not suitable for analysis and only one example shown?

We have deleted Figure 5A and added a figure as supplementary material showing the western blot analysis of the 35 breast tumours. Among the 40 tumour samples not suitable for analysis, there are three cases for which we were not able to detect a signal for the loading control (α-tubulin) or any other tested protein (i.e. actin, BRCA1, menin), while in the remaining 37 cases, bands for all these proteins were up-shifted for a reason that we were not able to discover, making the interpretation impossible.
Breast tumours are non-homogenous in nature. They include inflammatory and vascular elements but most significantly (by proportion) connective tissue components. The proportions of these components vary according to tumour type and sample type and also across a single tumour. Despite the fact that surgical samples used for research are selected among those with the highest proportional malignant cell content, some heterogeneity of tumour content remains from one specimen to the other. Furthermore, we would like to stress out that most breast tumours are very small when they are surgically removed and therefore, the material available for research is usually not abundant, which makes Western blot analysis difficult.

How were BRCA1 presence and absence defined? How were intermediate cases graded?

The presence and absence of BRCA1 was assessed using the GelDoc™ XR+ Imager (Biorad) and Image Lab™ software based on α-tubulin signals (both tyrosinated and detyrosinated). We scored only two situations, presence or absence, without grading the intensity of the BRCA1 signal, as we believed that this approach was less likely to generate biases given that protein quantification by Western blot analysis, even when using software tools as here, is not as sensitive as DNA or RNA quantification.
We indicate our assessment of the BRCA1 status in Table S4 of Supporting Information.

There is no statistical significance test performed on the data in figure 5B and no attempt at formal correlation between BRCA1 loss and miRNA expression, which limits interpretation.

We did perform a Pearson's chi-square test to challenge the significance of our observation, and we mention the *P* value of this test in the result section. This value is now also shown in Table 1.

7. Figure 6: Relationship between miRNA - 146a/b-5p expression and hormonal status: The comparison of expression median in triple negative with all tumours is not relevant. It would be more relevant to show the comparison of triple negative to non-triple negative tumours.

Figure 5 (previously Figure 6) now shows comparison of triple negative versus non-triple negative tumours.

It is not clear what the association with hormonal status adds to the overall message of the paper.

As mentioned in our manuscript in the abstract and the introduction, breast cancers arising in women carrying a *BRCA1* mutation are mostly classified as “triple-negative”, lacking expression of the estrogen receptor, the progesterone receptor, and the *HER2* gene. Conversely, sporadic triple-negative breast cancers have a high frequency of molecular events resulting in BRCA pathway

dysfunction ("BRCAness"). Therefore, triple-negativity correlating with BRCAness, the association of miR-146 expression with hormonal status in breast tumours for which it is not possible to determine if BRCA1 is present or not by lack of available tumour material, is highly relevant. As expected, miR146a and miR146b-5p are most expressed in triple negative breast tumour.

8. Page 3 - line 2: Please provide a reference for the first statement of the introduction.

Page 3 - line 5: Please reference a reference for "they account for no more than 1-2% of all breast cancer cases".

References have been added for the first statement of the introduction « Women with a germ-line mutation in the ubiquitously expressed *BRCA1* gene have a highly increased risk of developing breast and ovarian cancers. » (reviewed in Mavaddat et al, Molecular Oncology, 2010), and for the statement further down "...they account for no more than 1-2% of all breast cancer cases" (Anglian Breast Cancer Study Group, British Journal of Cancer, 2000).

2nd Editorial Decision 28 February 2011

The paper has been re-reviewed by one original referee with minor comments.

No further revision was requested.