Author's response to reviews

Title: Loss of miR-133a expression associated with poor survival of breast cancer and restoration of miR-133a expression inhibited breast cancer cell growth and invasion

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Version: 2 Date: 6 November 2011

Author's response to reviews: see over
Re: MS: 8203712595721335

Dear Editor:

Thank you very much for the reviewers’ constructive and insightful comments on our revised manuscript 8203712595721335. We have made the following changes to address the reviewers’ comments. The changes in the text are underlined and in red text.

Reviewer: Naohiko Seki

Reviewer’s report:

Major comments

Comment 1

The weak point of this article is not described the reasons why author selected microRNA-133a in this study. It is well known that miR-1-1/miR-133a-2, miR-1-2/miR-133a-1, and miR-206/miR-133b are clustered on three different chromosomal regions in the human genome, 20q13.33, 18q11.2, and 6p12.1, respectively. The miR-206 is similar to miR-1 in terms of expression and function but differs from the miR-1 sequence by four nucleotides. miR-133a-1 and miR-133a-2 possess identical mature sequences. miR-133b differs from miR-133a by a single nucleotide at the 3’ end. Based on these facts, I require an accurate mention and discussion in this article. The author must comment on the legitimacy that studied miR-133a in this study.

We fully agree and added such discussion in the introduction and discussion sections accordingly. In our previous study of screening miRNAs expression in breast tissue specimens using Exqion LNA™ microRNA Detection Probes by in situ hybridization, we observed miR-133a, not miR-133b or others, was significantly downregulated in breast cancer and correlated to tumor progression and patient survival.

Comment 2

The author described that FSCN1 as a target gene of miR-133a by bioinformatical analysis using TargetScan database. It is very difficult to search for FSCN1 as a miR-133a target gene by TagetScan database only. When an author finds FSCN1 in reference to past articles, it is necessary to list it precisely. The reason why FSCN1 comes up in this article is uncertain.

We fully agree. TargetScan database showed a large number of genes that are potentially regulated by miR-133a. The reason for us to focus on FSCN1
gene was because i). alteration of FSCN1 protein has been implicated for breast cancer invasion and metastasis and ii). FSCN1 was reported to be a target gene of miR-133a in other human malignancies. Now we have added such references in the result section.

Comment 3
At all unfortunately, the functional analysis of FSCN1 is not accomplished in this study. This thing lowers the value of this article. The functional analysis of FSCN1 (for example using siRNA methods) is possible by a method like the miR-133a analysis. The functional analysis of FSCN1, cell proliferation, invasion and migration assays, were required in this study.
We understand the reviewer’s concern. However, the function of FSCN1 in breast cancer cell proliferation, invasion and migration has been widely reported before. But now we have added co-transfection of miR-133a ASO and FSCN1 siRNA in transwell assays to demonstrate the function of miR-133 in suppressing cell migration and invasion is through targeting FSCN1.

Comment 4
This article described that reduction of miR-133a was associated with high clinical stages and relapse-free survival of patients with breast cancer. However, the number of patients of stage 1 is 0 in Table 2. In relapse-free survival analysis, the patients with the lymph node metastasis included. You should analyze it in patients without the lymph node metastasis by this analysis.
We re-analyzed the data accordingly.

Minor comments
There are not control experiments (without control oligonucleotide) in all transfection experiments. The cancer cell lines often cause some changes by control microRNA transfection.
We fully understand the reviewer’s concern. However, research in this area generally agrees that miRNA gene transfection is usually not using any mock controls. In this study, we just used vector-only as a control, which may be considered as the mock control since the vector-only also contains a non-specific sequence.

You should show the negative or positive expression of miR-133a in figure 1 A-C by in situ analysis of the specimens of breast cancer patients.
We agree and added them accordingly.

A mention is vague “benign breast disease”.
The benign breast disease is referred as some breast diseases that will not be subjected to malignant transformation into breast cancer, which were used as controls.
There is not mention of the specimens figure 1D.  
We fixed it accordingly.

There is not a mention why author analyzed the MCF-7 cell line only.  
MCF-7 cell line is one of most studied breast cancer cell line, which was also used in this study. We fully understand the reviewer’s concern and add another breast cancer cell line, MDA-MB-231 cells, for functional analysis.

Reviewer: Hailong Wu  
Reviewer's report:  
Major Compulsory Revisions  
1. In this paper, the authors demonstrated that mir-133a expression reduced in breast cancer cell lines by comparing mir-133a expression among HBL-100, ZR75-1, SKBR3, T47D, MCF-7 and MDA-MB-435. However, both HBL-100 and MDA-MB-435 have been classified as misidentified cell lines by ATCC because of presence of Y chromosomes and cross-contamination with the M14 melanoma line respectively.  
The link to support my review: http://www.atcc.org/CulturesandProducts/CellBiology/MisidentifiedCellLines/tabid/683/Default.aspx  
Since the conclusion of lower mir-133a expression in breast cancer was accounting to the mir-133a level in HBL-100, it's better to replace HBL-100 to a real human mammary epithelial cell line, like HMEC.  
Many thanks for the valuable information and removed these two cell lines, but add HMEC and MDA-MB-231 cells for qPCR analysis of mir-133a expression.

2. MCF-7 is well known to have non-invasive property. I don't expect to see a lot MCF-7 cells in invasion assay. The authors need use an invasive breast cancer cell line, such as MDA-MB-231, to perform invasion assay.  
We fully agree and added MDA-MB-231 cells in such assay.

3. The pictures of wound healing assay should be re-taken because the cell morphology is not distinguishable.  
We replaced with better imagines.

4. Xenograft model is required to make this paper accept.  
We fully understand the reviewer’s concern for animal experiments. This study was just a proof-of principle study. Addition of xenograft assay will not mechanistically answer the question for the role of mir-133a in FSCN1 knockdown and in breast cancer. In addition, xenograft experiment needs more time to get ACUF approval and perform the experiments. We will leave it
to the future study.

5. I don’t understand why they reverse transcribed RNA into cDNA before detection of mir-133a expression by using TaqMan MicroRNA Assay kits from ABI. This kit starts directly from total RNA, and cDNA synthesis is not required. Please clarify it.

We used the TaqMan ® MicroRNA Assays (ABI) to quantite the expression of miR-133a. This kit includes two-step protocol which is the reverse transcription with a miR-133a primer and followed by real-time PCR with miR-133a probes. The information about the kit is 1. RT2246, has-miR-133a, lot:1004074-HG6; 2. TM2246, has-miR-133a, lot:1004074-HG6.

Also could be found in the website of ABI blow.

6. ISH method detail is too simple.
We revised it accordingly.

Minor Essential Revisions
1. The authors need show ISH data on positive control and negative control.
We added it accordingly in the supplementary data.

2. In the second paragraph of Background, ‘is highly expression’ should be ‘is highly expressed’.
We corrected it accordingly.

Discretionary Revisions
1. It’s better to use anti-mir-133a oligo in luciferase assay
Great suggestion, but it may be difficult to generate data we would like to have.

2. It’s better to show FSCN1 expression level in breast cancer cell lines corresponding to mir-133a level.
We fully agree and added it accordingly in Fig 1E.

Reviewer: Junfang Ji

Reviewer’s report:
In this work, Lee et al provided data demonstrating that 1) the expression of miR-133a was gradually reduced from normal through benign to cancerous breast tissues; 2) the reduced miR-133 was associated with short relapse-free survivals; 3) miR-133 could suppress cell growth, cell migration and cell invasion; 4) FSCN1 is the potential target gene of miR-133. According to these
results, the authors concluded that loss of miR-133a expression associated with the poor survival of breast cancer and restoration of miR-133a expression inhibited breast cancer cell growth and invasion.

Although the data are interesting, this paper appears not to represent a strong candidate for publication in BMC Cancer yet. To increase the quality of this paper, some concerns might be addressed.

1. Major Compulsory Revisions
a. The survival analysis was only done in one cohort. The further validation is required in an independent cohort to draw the conclusion.

   We fully understand the reviewer’s concern. It will be great to include an additional patients’ cohort, which will be our future study.

b. This paper showed the function of miR-133 in suppressing cell migration and invasion and miR-133 targeting FSCN1 disjointedly. To increase the logic and rationality of this miR-133 functional part, it will be better to examine and to show 1) the linear correlation of miR-133 expression and FSCN1 level in the clinical samples and cancer cell lines; 2) whether the function of miR-133 in suppressing cell migration and invasion is through targeting FSCN1.

   We fully understand the reviewer’s concern. For the first query, it will be very difficult to get the data. The reason is simple: it is not possible or very rare that a gene is exclusively regulated by another gene. Often it is regulated by different genes to make biology very complicated. For the second query, we fully agree and added such data to satisfy in Fig 3E.

c. The novelty of this paper seems to be weak. It has been shown that miR-133 is down-regulated in breast cancer (Iorio et al, 2005) and FSCN1 is the target gene in esophageal squamous cell carcinoma and bladder cancer (Kano et al, 2010; Chiyomaru et al, 2010). It will be good to interpret the rationality of choosing miR-133a as the object to work with.

   We fully understand the reviewer’s concern. Our current study reported data on breast cancer but not repeat in other cancers. It is the first study in breast cancer to link altered expression of mir-133a and FSCN1, which is novel. In addition, FSCN1 protein was implicated in breast cancer invasion and metastasis and our study will provide very insightful information in this matter.

2. Minor Essential Revisions
a. The description in the text (line 13-14 at page 14) and the labeling in the figures (Fig 2A, 2B) are inconsistent. In the text part, it wrote that the growth inhibition were 0.8%, … and 18.7 for 60h, 84h, 108h and 132h after gene transfection. However, the figures showed that the growth inhibition assay was done at 24h, 48h, 72h and 96h after inoculation. This needs to be clarified.

   We apologize for the confusion concerning Figure 2A and 2B. The data was
correct. In the MTS assay, MCF-7 cells were first transfected in 6-well plates and then were harvest and sub-cultured in 96-well plate for up to 96h. The labels of 24h, 48h, 72h and 96h in Figure 2A and 2B mean the time points in 96-well plates. Actually it is 60h(36h+24h), 84h(36h+48h), 108h(36h+72h) and 132h(36h+96h) after transfection. Now we revised them accordingly in Figure 2A and 2B.

b. In Figure 1D, the expression of miR-133a in fresh breast cancer and benign diseases specimens was quantited relatively to U6 expression. Each specimen was performed in triplicate. So both the group of breast cancer (18 cases) and group of benign diseases (10 cases) have the mean and SD values of miR-133a expression. In the figure 1D, the black line in the bar means the mean value and the error bar means the SD value of each group.

c. In line 4-5 of page 3, “…miR-133a can suppresses tumor cell invasion and migration potential and targeted expression of FSCN1…” should be “…miR-133a can suppress tumor cell invasion and migration and target the expression of FSCN1…”. We revised it accordingly.

d. In line 2 of page 9, “…the manufacturer’s protocol.. The miR-133a…” should be “…the manufacturer’s protocol. The miR-133a…”. We revised it accordingly.

e. In line 11 of page 13, “…Furthermore, , we…” should be “…Furthermore, we…”. We revised it accordingly.

Thank you again for your consideration for publishing our manuscript in BMC Cancer.

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