Author's response to reviews

Title: Epigenetic silencing of miR-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R

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Version: 4
Date: 10 February 2014

Author's response to reviews: see over
February 10, 2014

Editor

BMC Cancer

Dear editor,

As permitted by your letter of January 28, 2014, we are submitting the revised manuscript entitled “Epigenetic silencing of miR-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R” (MS ID: 9568184951082539). On behalf of all authors of the manuscript, we would like to show you our gratefulness for the important suggestions in improving the manuscript. Attached please find a point-by-point response to the reviewers’ criticisms for the previous version of the manuscript. In addition, we have removed the image of microarray cluster analysis (previously Fig. 1C), given that the entire microarray data have been submitted to the public database Gene Expression Omnibus (GEO, assigned accession #: GSE47011).

Thank you very much for taking time to process this manuscript. We look forward to further correspondence and discussion with you concerning the current version of the manuscript.

Sincerely yours,

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Response to reviewers’ comments

Reviewer: Dr. Bolin Liu

This study aims to determine the basis of IGF-1R upregulation in trastuzumab-resistant breast cancer cells. Mechanistically, upregulation of IGF-1R in the resistant cells is due to the epigenetic silencing of a tumor suppressive miRNA, miR-375 which has been shown to target IGF-1R mRNA. The inverse correlation of miR-375 expression and IGF-1R mRNA levels has also been identified in clinical samples of breast cancer patients. The results implicate miR-375 as a potential target in combination with trastuzumab for the treatment of HER2-positive breast cancer. The information included would be very interesting to those investigators who are exploring the mechanisms of trastuzumab resistance.

>> Thank you very much for the favorable comments on our manuscript.

Minor Essential Revisions:

1. On page 14, it says “Next, we investigated whether miR-375 suppresses trastuzumab resistance and metastasis by targeting IGF1R.” However, there is no study related to tumor metastasis. And the following sentence “Concurrent with the expression pattern of miR-375, IGF1R protein and mRNA levels were lower in trastuzumab-resistant cells than parental SKBr-3 cells (Figure 3A).” is a wrong statement.

>> We used to assess a potential role of miR-375 in the metastasis of trastuzumab-resistant breast cancers during the phase of manuscript preparation. However, we later realized that the relationship, e.g. the molecular connection, between trastuzumab resistance and metastasis can be very complicated and is beyond the aim of the current study. We are very sorry for failing to exclude the statement related to cell invasion or breast cancer metastasis in the previous version of the manuscript, and have done so in the revised manuscript.

Sorry again for the completely wrong description that “Concurrent with the expression pattern of miR-375, IGF1R protein and mRNA levels were lower in trastuzumab-resistant cells than parental SKBr-3 cells (Figure 3A)”. We have now corrected the statement.

2. Fig. 2B, the legend in “Parental” is wrong.

>> Indeed, the inhibitor control and miR-375 inhibitor transfection groups in treatment of parental SKBr-3 cells were wrongly labelled, which has now been corrected. We are very sorry for the careless omissions.

3. Herceptin induced substantial apoptosis in the parental SKBR3 cells (fig. 2D) and BT474 and MDA-MB-453 cells (fig. 2G). These data are not in agreement with a number of literatures indicating that Herceptin mainly inhibits cell proliferation via induction of cell cycle G1 arrest without apoptosis.
Trastuzumab treatment of breast cancers was documented to trigger G1 arrest in the cell cycle (Le et al, Cell Cycle, 2005; 4: 87-95). However, trastuzumab was also reported to facilitate apoptosis of breast cancers (Mohsin et al, J Clin Oncol, 2005; 23: 2460-8; Hudis CA, N Engl J Med, 2007; 357: 39-51). Given the crosstalk between molecular machineries of cell proliferation and survival in various cells including trastuzumab-resistant cells (Scaltriti et al, Proc Natl Acad Sci U S A. 2011;108: 3761-6), we hope you agree that elevated apoptosis of neoplastic cells also contributes to the anti-tumor activity of trastuzumab.

4. It is not clear whether the clinical samples used in fig. 3D are from all breast cancers or HER2-positive breast cancers.

Since it is very difficult to obtain trastuzumab-resistant breast cancer samples, we collected clinical samples from 40 breast cancer patients, among which 17 was confirmed HER2-positive (immunohistochemical immunohistochemistry score 3 or fluorescence in-situ hybridisation positive) whereas others were HER2-negative. We have now supplemented the materials and methods with the above information for clinical sample collection. Thank you very much.

5. Fig. 4C shows survival curves with Kaplan-Meier analysis in the animal studies. What does the data mean?

In Fig. 4C, SKBr-3 cells modified to express miR-375 or a miRNA control was used to inject nude mice for generation of an s.c. xenograft tumor model. This was followed by treatment of the mice with trastuzumab. The prolonged survival of mice bearing miR-375-expressing tumors suggests that miR-375 plays a role in sensitizing breast cancers to trastuzumab treatment. We hope these results help verify in vivo tumors the involvement of mir-375 in regulating the response to trastuzumab treatment by confirming that mir-375 restores trastuzumab sensitivity of cultured SKBr-3 cells by targeting IGF1R.

6. The quality of western blot data (fig. 3A and fig. 5E) needs to be improved.

Thank you very much for the suggestion. We agree that the quality of these blottings need to be improved, and has thus replaced the blottings of cyclin D1 and AKT in Fig. 5E with images of replicated blots.

Reviewer: Dr. Ratna Vadlamudi

In this study, authors examined the mechanisms that contribute to trastuzumab resistance of breast cancer cells. Using acquired resistance model cells, and whole genomic miRNA profiling, authors identified alternations in the levels of 9 miRs. In this study, they focused on the hypothesis that miR375 levels (one of the nine miRs identified in the screen) contribute to up regulation of IGF1R conferring trastuzumab resistance. Using biochemical, and reporter gene assays, authors demonstrated that miR375 indeed target IGF1R. Further, ectopic expression of miR-375 inhibited
IGF1R expression, restored sensitivity to trastuzumab. Some evidence was also provided demonstrating genetic regulation of miR375. Utilizing 40 breast tumors samples, authors found the levels of miR-375 were inversely correlated IGF1R in clinical samples. Overall this is well designed study and results have implications for targeting trastuzumab resistance.

1. Page 10: This statement need to be corrected: “and had a significantly higher proliferation capacity in an MTT assay (p<0.05, Figure 1B)”. MTT assays presented do not measure cell proliferation rather they provide a relative measure of total viable cells.

>> We agree that MTT assay measures total viable cells, the result of which reflects the combined activity of cell survival and proliferation. Therefore, we have revised the description accordingly in the updated manuscript. Thank you very much.

2. Figure 3D. More information on the type of breast cancer tissues (n=40) used for this analysis (tumor type, status of HER2, whether tumors were treated with any HER2 targeting drugs, therapy response if available) need to be included in the methods, results and figure legend.

>> Sorry for not providing enough information for clinical samples in the previous manuscript. As stated above, we collected clinical samples from 40 breast cancer patients, among which 17 was confirmed HER2-positive (immunohistochemistry score 3 or fluorescence in-situ hybridisation positive) whereas others were HER2-negative. We have now supplemented the materials and methods with the above information for clinical sample collection. Thank you very much.

3. Fig 5 C, D. The location of amplified region in the miR-375 promoter need to be indicated as well as the rationale for testing this specific region in the promoter and key transcriptional elements etc. should be included.

>> Thank you for the important suggestions. In Fig. 5 C and D, we amplified the genomic fragment 40 to 560 bp upstream of the pre-miR-375 coding sequence, which covers a large proportion of the reported promoter region of both mouse and human pri-miR-375 (Avnit-Sagi et al, PLoS One, 2009; 4: e5033). The methylation-specific PCR was designed to measure the DNA methylation of 2 CpGs 60 bp upstream of the pre-miR-375 coding sequence. The above information has been supplemented in the revised manuscript.

4. Fig 5E. The quality of Western in Fig 5E is poor. Better quality images from new experiment should be included.

>> Thank you very much for the comment. We know that the blottings of cyclin D1 and AKT in Fig. 5E need to be improved, and has thus replaced it with images of replicated blots.

5. Authors presented no data on Invasion and metastasis. Therefore the statements in results and
conclusion section refereeing to effect of miR-375 on invasion and metastasis should be deleted from text. Some examples: Page 4: Ectopic expression of miR-375 inhibited IGF1R expression, restored sensitivity to trastuzumab and suppressed tumor cell invasion. Page 12: Trastuzumab-resistant breast cancer cells exhibit survival and invasion advantages over parental cells; Page 14: we investigated whether miR-375 suppresses trastuzumab resistance and metastasis by targeting IGF1R.

>> Thank you very much for the criticism. We agree that the present study was mainly designed to assess the role of miR-375 in trastuzumab resistance, and have deleted all descriptions concerning the invasion or metastasis of breast cancers.