Author's response to reviews

Title: Kif2a Silencing Inhibits the Proliferation and Migration of Breast Cancer Cells and Correlates with Unfavorable Prognosis in Breast Cancer

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Author's response to reviews: see over
Dear editor:

We hereby resubmit our revised manuscript by “Wang, et al” for consideration of publication in the BMC Cancer.

We would like to express our sincere appreciation to your constructive comments. Your comments are helpful to improve our manuscript. We have revised the manuscript accordingly by track changes and a point by point response is given in below. All of our authors have read the revised manuscript and agree with the revision.

We hope our revised manuscript is acceptable to you. We look forward to hearing from your positive decision!

Sincerely yours

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Response to reviewer Brigitte L. Theriault
Many thanks to your valuable comments! We have carefully read your excellent comments, and we have answered questions you raised as:

Comment 1. The authors did not specify how the patient tumors were staged or graded, or who performed that determination. Was a pathologist involved in reviewing the cases for inclusion in the study? If so, the role of this expert should be acknowledged in the manuscript. Also, how was the patient data reviewed to ensure that only patients with complete follow-up (overall survival times) were included in the study?
Response:
Tumor grade is based on the criteria of WHO Classification of Tumors of the breast (The Fourth Edition, 2012) with consideration of three indicators listed in below;

a. The most obvious glandular tube (>75%) +1; moderately differentiated glandular tube (10-75%)+2; and solid block or cord (<10%)+3.
b. The nuclear size, shape and chromatin: consistent for +1; moderate irregular for +2; pleomorphism for +3.

c. Nuclear mitosis (field diameter 0.40mm, 40x objective lens): ≤ 4/10HPF for +1; 5~9/10HPF for +2; ≥ 10/10HPF for +3.

The sum score of three indicators is the final score for each patient: 3~5 for grade I (well differentiation), 6~7 for grade II (moderate differentiation), 8~9 for grade III (poor differentiation). This is added to page 5 line 1-2.

A pathologist from Qilu Hospital did the diagnosis. Sorry of our oversight, her name (Qinghui Zhang) is added to the acknowledgment now. Thanks for the reminder.

In this study 120 out of the 189 patients, have been followed-up completely over the whole course. It was these 120 patients that we did Kif2a analysis. This is added to page 5 line 5-6.

Comment 2. The authors also describe performing IHC on patient tumor slides. How were the slides/tissue sections selected for inclusion in the study? What were the criteria (% viable tumor cells, presence of adjacent normal tissue, the number of tissue sections stained/patient) on which the authors based the selection of tissue sections for staining? Was a pathologist involved in the selection of the tissue sections?

Response:
IHC was performed on slides from the same block as the pathology diagnosis. The tumor section was trimmed carefully to avoid necrotic tissue and normal tissue presence on slides, whilst the adjacent normal tissue was sampled at least 1 cm distance from the tumor to ensure no tumor tissue is included in the slide. Yes, the same pathologist was involved in the selection. This is added to page 6 line 20-21.

Comment 3. With respect to the IHC staining, representative images of the different intensity categories for the Kif2a staining should be shown as a reference in the supplementary materials section. The pathologist involved in scoring the IHC sections and should also be acknowledged in this study (page 14, acknowledgment).

Response:
The intensity categories of IHC staining is shown in the supplementary Fig 1. The same pathologist was involved in the scoring.

Comment 4. Details are lacking on many experimental procedures, including: a) Reference to cell culture procedures is not included. b) How much total protein was loaded onto the protein gels? c) GAPDH antibody dilutions, antibody supplier information, antibody incubation times, and how the chemiluminescence signal was detected (film, scanner)?d) How the protein signal intensity was quantified (image analysis software), and how many replicates were included in this analysis.

Response:
Cell culture procedures reference is added to Page 7 line 20-22, Page 8 line 1. 50µg was loaded onto protein gels. This information is added to Page 6 line 12.
The dilution of GAPDH was 1:3000. The incubation was overnight at 4 degree C. The chemiluminescence signal was detected by scanner using Bio-Rad ChemiDoc™ Image LabSoftware. The protein signal intensity was quantified by JD801 Analysis software (Jie Da Science and Technology Development Co., Ltd. Jiangsu province, China) and replicated three times. The information is added to Page 6 line 13-17.

Comment 4. e) It is unclear whether the authors performed real-time PCR of Kif2a expression, or RT-PCR and gel electrophoresis. Please clarify. If real-time PCR was performed, details on the methodology (SYBR green or TaqMan probes?), or how the mRNA expression level was calculated (fold change, or intensity levels?) need to be specified. If RT-PCR and gel electrophoresis were performed, how was the intensity of the bands determined?
Response:
We performed RT-PCR for the expression level of endogenous KIF2A mRNA in MCF-7, MDA-MB-231, T47D and MDA-MB-468 breast cancer cell lines (Page 8 line 3-10). We also performed real-time RT-PCR of KIF2A expression on breast cancer tissues. We have added these details on Page 5 line 14-19. The mRNA expression level of KIF2A was calculated (real-time RT-PCR) using fold change (Page 6 line 7-8). JD801 Analysis Software was used to determine the intensity of RT-PCR gel electrophoresis band (Page 8 line 7-8).

Comment 4. f) P-value in Figure 1B cannot be 0 – please include a number. g) Many figures do not have a P-value stated either in the figure, or the figure legend. h) No scale bars are included in any of the figures to indicate that the images shown were taken at the same magnification. Especially for Figure 2, where A-B, C-D, and E-F look like they were all taken at different magnifications. i) The images in Figure 2 E and F are of very poor quality, and it is virtually impossible to see the FISH probe signals.
Response:
P<0.001 is added in Figure 1B.
We have added P-value number in all figure legends or figures with your suggestion (Figure 1, 3, 4 and their legends in Page 20-22, and also in manuscript Page 10 line 16; Page 11 line 10).
We have added scale bars wherever requires including Fig 1, 4. Figure 2 has been removed following other reviewers comments.

Comment 4. j) Figure 5 was provided in a very poor quality format - the reviewer could therefore not appropriately evaluate the data presented in this figure. k) Details are missing on how the cells were counted for the migration/invasion assays how many fields were counted within a filter? How many replicates were included in the experiment? Were the authors blinded? l) images of migration assays need to be included.
Response:
We have improved the quality of Figure 5 (now Figure 4 following the removal of Figure 2). P-value and scale bars have been added, too.
In migration and invasion assays, we counted 6-field (200x), blinded. All experiments were done in triplicate. This is added to Page 9 line 13-14.
The images of migration assay are added as Fig 4, 4D-F.

Comment 5. The authors state that two siRNA molecules targeting Kif2a were employed in their analyses, giving the same results. However the authors chose to only show the data for one siRNA molecule. In order to strengthen their postulate, the authors should show the data on both siRNA molecules (perhaps as supplementary data), as this would further support their conclusions of the effect seen in response to Kif2a knockdown.

Response:
PCR data of the kif2a expression for both siRNA molecules are included. In supplementary Figure 2. We also made slight change in the text to improve the imprecision, i.e. the two siRNA molecules had similar effects (Page 8 line 14).

Comment 6. The authors state that Kif2a expression correlated with poor prognosis in breast cancer patients. The only data shown to support this postulate are the Kaplan-Meier curves in Figure 3, and Odds ratios stated in the text. However, this data is poorly described, and insufficiently analysed to support the authors’ conclusions. The authors should present a graph showing the Kif2a IHC expression levels of each patient, and indicate where the cutoff was chosen for the dichotomization between the high and low expression groups, along with the statistical analyses. The results from the prognostic analyses (including univariate, multivariate, and correlational analyses with clinical variables vs. months survival and survival rates between groups, odds ratios or hazards ratios, and correlation coefficients with confidence intervals and associated P values) should be included in a table format in the results section of the manuscript.

Response:
The comment requesting a graph is not entirely clear to us, but we have addressed this by adding a box-plot figure showing that KIF2a expression in breast cancer was higher than in the adjacent tissue in the supplementary Figure 3. We have also added more detailed results summarizing the univariate, multivariate, and correlational analysis with clinical variables and survival outcomes in table 2, also in Page 11 line 16-21. We trust these are sufficient to satisfy the reviewer.

Response to reviewer Pauline Funchain:
Many thanks to your comments! We have revised our manuscript with your comments as below:
Major compulsory revision
Comment 1. The discussion in the second to last sentence of the first results paragraph (pg8) refers to a p-value for a Western blot but there is no quantitation shown. Please add a numerical table or graphical plot that justifies the p-value.
Response:
We have added Figure 1D (including P-value) to address this issue.

Comment 2. KIF2A expression is more appropriately analyzed with multivariate analysis in
Multivariate regression analysis was conducted for the results presented in Table 1. We also added the multivariate analysis and result in the abstract in Page 2 line 10, 17-18.

**Comment 3.** Figure 2 showing HER2 expression is unnecessary. Please remove.

Response:
Figure 2 is removed.

**Comment 4.** The last sentence of the first paragraph of page 9 (as well as the last sentence in the first paragraph of the discussion and the 4th line on pg12) refers to the frequency of overexpression of KIF2A in patients with lymph node metastasis and states the p-value is significant but does not provide any numbers regarding frequency. Please provide the observed frequencies to provide a basis for the p-value.

Response:
The overexpression of KIF2A in patients with lymph node metastasis was more frequently observed in patients with lymph node metastasis than those without lymph node metastasis (6.52±1.01 vs 5.91±0.78, P<0.001). This is added to page 11 line 8-10. The expression of KIF2A in cancer had been shown in Table 1.

**Minor essential revisions**

**Comment 5.** The authors should take care to correctly refer to human proteins in all caps (e.g. KIF2A) and genes should be both italicized and all caps.

Response:
We have changed all the kif2a into KIF2A. And genes have been both italicized and all caps in Page 5 line 19-20, 22; Page 6 line 1; Page 8 line 13.

**Comment 6.** Small English revisions: pg 3, mid page: “2.95 folds” should be “fold”; pg 3, last paragraph, "adjacent epithelium tissues" should be "adjacent epithelial tissue"; multiple places with spacing mistakes e.g. “tissues (Fig.1E)” should be “tissues (Fig. 1E)”

Response:
We have changed 2.95 folds into 2.95 fold in Page 10 line 14; adjacent epithelium tissue into adjacent epithelial tissue, Page 10 line 20; tissues(Fig.1E) into tissues ( Fig.1E), Page 10 line 19, etc.

**Comment 7.** Multiple grammatical errors in the discussion-please check that verbs agree in number with subjects and other including MDA-MB-231 is referred to as a breast cancer cell, rather than a cell line.

Response:
We invited a native speaker to correct grammatical error in the manuscript. MDA-MB-231 cell line is referred to as MDA-MB-231 breast cancer cell line (Page 12 line 7).

**Discretionary Revisions**
The paper would be stronger if data were shown with a second, confirmatory breast cancer cell line.

Response:
Thank you for your suggestion.