Author’s response to reviews

Title: AKAP3 correlates with triple negative status and disease free survival in breast cancer

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Author’s response to reviews: see over
Dear Editor

I thank the reviewers for these thoughtful suggestions. We have revised the paper as follows:

**Reviewer number: 1**

**Major Compulsory Revisions**

1- Percentage of cancer cells in tumor samples (cellularity) should be determined to be sure that loss of AKAP3 mRNA expression is not simply due to low content of cancer cells in each sample.

   • The content of cancer cells in each sample was pathologically checked before including in Biobank. The material and methods were changed accordingly.

2- Internal controls of real-time PCR are lacking. Data obtained from AKAP3 qPCR need to be normalized relative to at least two endogenous genes (for instance RPLP0, EEF1G or PP1A), in order to account for extraction, reverse transcription and possible degradation of RNAs. In absence of these controls, qPCR cannot be quantified and results are not conclusive.

   • The referee is right to point out the number of internal control in Real-Time PCR and authors have published a paper about this issue previously and it is important when the fold change of expression is reported[1]. But, the significant results reported in this study was seen when AKAP3 was not expressed and AKAP3 negativity is a real absence of it and it is not based on the specific cut point. So it seems that the number of housekeeping would not influence the results. Moreover RNA quality and quantity was checked before the experiment and Actin Beta was detected in all samples. A sentence was added to the result to make it clear in this part in line 140.

3- An additional Table should be provided indicating AKAP3 expression levels (delta-CT and normalization relative to endogenous genes) in each sample analyzed (tumor and adjacent) and mentioning corresponding clinical data (age, size of the tumor ER, PR, HER2, treatment, time of survival etc).

   • Table 2 was provided according to reviewer comments in order to show AKAP3 expression in tumor and normal tissues and clinico-pathologic features of the tumor. The presented significant result was based on negative and positive expression of the AKAP3.
4- A Figure should be provided (scattered dot plot) comparing AKAP3 expression (normalized to endogenous genes) in each tumor and adjacent tissue (classified according to grade, ER positivity, molecular subtype, treatment, etc).

- Since the presented significant result was based on negative and positive expression of the AKAP3, the figures were provided with dichotomized results (Figure 2).

5- Figure 1 is of very poor quality. What does neg-censored and pos-censored mean? Furthermore there is a problem with p values in left and right panels, as these seem to have been inverted. This may lead to false interpretation of the results.

- Figure 1 was re-prepared with higher quality, p values were corrected. Censoring was clearer in Figure legend. The authors wish to prepare it based on the journal desired criteria.

6- 5-year survival for patients from different groups expressing low or high levels of AKAP3 (cut-off defined as >2 fold or < 2 fold that of median value for normal breast tissue).

- According to the previous comment, the significant results were seen according to the real absence or presence of AKAP3. So, the survival curve can be prepared with negative and positive groups.

7- On line 140, authors write: “There was no association between AKAP3 expression in normal adjacent tissues and triple negativity, but there was a significant association between lack of AKAP3 in normal adjacent tissue and poor prognosis (p=.003)”. These two statements are contradictory and should be discussed in more details, as triple-negative breast cancer is a subtype of poor prognosis. Examination of Figure 1 suggests inversion of p values between adjacent tissues (left) and tumor samples (right), or an error in statistical analysis.

- We appreciate the chance to make ourselves clearer. It is discussed in more details in lines 151-157. The authors wish to explain it completely. The referee is right for inversion of p values in Figure 1, it was corrected.

8- On line 212, authors write: “AKAP3 may act as inhibitor of proliferation since it was not expressed in higher stage and tumor size”. This conclusion is over-interpreted, as down-regulated genes are not all involved in inhibition of proliferation.

- It was a suggestion for the AKAP3 role and the discussion was modified, Line 194-196.
Minor Essential Revision:
1. On line 298: Table 1 and not table 1.
   - It was corrected.

2. ACTB gene: full name should be given (actin beta)
   - Full name of Actin Beta was included for first time with abbreviation in parenthesis, line 94.

Reviewer number: 2

Major points:
1- The number of samples mentioned in the abstract is not the same as mentioned in the materials and methods section (165 and 164, respectively). So, which number is the exact number of samples used? Moreover, it seems that the authors add up the two cell lines used as breast tissue samples (line 73 of the manuscript document), while they are not. So, separation must be made between breast tissue samples and the cell lines, i.e. they do not add up to the tissues.
   - The number of samples was clarified and the separation between breast tissue samples and cell lines was made, line 74-76.

2- The authors used ER, PR, and HER2 status for the cases used. However, they did not mention, whatsoever, how and according to which guidelines these status have been determined. Data of cut-point of positivity, clones used for identification, and whether HER2 status was defined by IHC only or with other methods like FISH or CISH.
   - IHC explanation and guidelines for cut points was added to table legend.

3- The authors in their analytical plan pointed to the analysis of the PCR data with different combinations of ER, PR, and HER2 status (lines 111-112 in the document). So, the results of these analyses are to be provided in the results section and discussed accordingly.
   - A different combination of the subtypes were not resulted in significant output and just triple negatives had been significant. It was added in line 140-141.
4- A table summarizing the results of statistical associations between RT-PCR results and the clinic-pathological data should be provided.

- A table was provided according to reviewer as table 2.

5- The authors defined cases into positive and negative regarding expression levels of AKAP3. However, they did not mention whether negativity is a real absence of AKAP3 or this a dichotomization of AKAP3 RT-PCR data based on a specific cut point. Please, specify.

- AKAP3 negativity is a real absence of it and it is not based on the specific cut point. It was added in the results. Line 141-142.

6- The conclusion firmly states: "It was found that this relationship is originated from the difference in AKAP3 expression, not therapy distribution between two groups of patients". The authors here interpreted the associations they found in their study as a causation, which is not always the case. Please re-phrase with tone down of the study conclusions.

- It was a suggestion for the AKAP3 role and the discussion was re-phrased.

Minor points
1- Table 1 describing the clinico-pathological criteria of the studied patients/samples, and these data were verbally written in the beginning of the results section (lines 120-127). One form of data presentation is enough (whether the table or the detailed written description).

- Some of the written data was omitted and it can be followed in table1.

2- The survival curves need to be double checked as the lines representing positive and negatives appears to be more separated in the plot labeled as not significant (p=.8) and vice versa.

- Survival curve was checked and it was a mistake in writing the p values. It was corrected.

2- More attention is needed to the punctuation throughout the manuscript for better readability of the manuscript.

- Punctuations were reviewed and the authors wish to prepare it based on the journal desired criteria.
Editor's Request

The name of the ethics committee was included in line 85.

Reference:


Sincerely yours,

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