Author's response to reviews

Title: Prognostic significance and therapeutic potential of the activation of anaplastic lymphoma kinase/protein kinase B/mammalian target of rapamycin signaling pathway in anaplastic large cell lymphoma

Authors:

Ju Gao (gaoju651220@126.com)
Minzhi Yin (mzhuyin@hotmail.com)
Yiping Zhu (zhuyiping918@yahoo.com.cn)
Ling Gu (guling00@163.com)
Yanle Zhang (zhangyanle123@126.com)
Qiang Li (lqcm2000@yahoo.com.cn)
Cangsong Jia (jiacangsong@sina.com)
Zhiui Ma (ma_zg@yahoo.com)

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Author's response to reviews: see over
Response letter to the reviewers’ comments

Reviewer 1's report

Title: Activation of nucleophosmin-anaplastic lymphoma kinase/ protein kinase B/mammalian target of rapamycin signaling pathway in anaplastic large cell lymphoma and its correlation with the clinicopathologic variables

Version: 2 Date: 28 March 2013

Reviewer: Emmy D.G. Fleuren

Reviewer's report:

Thank you for the opportunity to review this manuscript. This is an interesting study linking ALK expression to activated downstream targets (AKT/mTOR) in ALCL. The authors also investigated possible correlations between expression of these proteins and patient outcome. There are however several concerns that need to be addressed prior to publication that will improve the quality of this work.

Major Compulsory Revisions:

1. Language corrections are necessary throughout the whole manuscript before it can be considered for publication. I have indicated the general problems and some (not all) specific examples below, but would highly recommend it to be fully edited by a native speaker. Without extensive editing, I’m afraid that it is hard for readers to go through the paper and fully understand the scientific content.

General problems:

- The words ‘the’ and/or ‘a’ are often missing
- Incorrect use of words
- Punctuation often incorrect
Some specific examples:
- Abstract; background: Activation of AKT/mTOR pathway -> Activation of the AKT/mTOR pathway or activation of AKT/mTOR
- Abstract; background: ‘from clinical perspective’ -> from a clinical perspective
- Abstract; background: associated -> # correlated
- Abstract; background: prognostic value -> # the prognostic value
- Background; 1st paragraph: accounts for up to 30% to 40% -> # accounts for up to 40% or accounts for 30% to 40%
- Background; 2nd paragraph: later -> # latter (also discussion; 2nd paragraph)
- Background; 3rd paragraph: While -> # Because the…, indicating -> # this indicates or The…, indicating
- Background; final paragraph: In present study -> # in the present study- Discussion; 1st paragraph: much in consistent to -> # correlated well; literatures
-> # literature
- Discussion; 2nd paragraph: . -> #.
- Discussion; 2nd paragraph: whole ALCL patients -> # all ALCL patients

I have revised the manuscript again and made all the corrections you indicated above. Also I asked a native-English speaker who is professional in molecular pathology to help me with language corrections. I have highlighted all the corrections in the manuscript.

2. Title: The authors state that the NPM-ALK/AKT/mTOR pathway is activated in ALCL. Although this is true for the AKT/mTOR pathway by showing p-AKT and p-mTOR expression in the ALCL cohort, it is not certain whether this is solely caused by (NMP/ALK) activation. Although the authors have indeed linked ALK expression to p-mTOR and p-AKT expression, they have not considered p-ALK levels. Since AKT and mTOR signal downstream of numerous other (receptor)proteins, these may also contribute and significantly correlate to


p-AKT/p-mTOR signaling. This concern should at least be addressed in the discussion section.

This is a good question! Yes, the AKT/mTOR pathway can be activated by kinases other than ALK, or mutation of some certain tumor suppresser genes, like PTEN. This could be proved by AKT/mTOR activation in some ALK- ALCL cases. To further prove that ALK fusion kinase could activate AKT/mTOR, we did an in vitro study to overexpress NPM-ALK in BaF3 cells. We demonstrated that a more hyper-activated AKT/mTOR pathway in BaF3 cells stably transfected by NPM-ALK than in BaF3 cells transfected with empty vector or the parental BaF3 cells.

Since all ALK fusion proteins generated by chromosomal translocations constitutively auto-phosphorylated, so expression of ALK indicates activation of the kinase. We do not routinely test ALK expression with a specific phospho-ALK antibody in ALCL, but ALK11 or ALK1.

3. Methods; immunohistochemical studies: In this section, the authors mention the use of two ALK antibodies (ALK11 and ALK-1). It is however unclear why they use/mention two different ALK antibodies, while in the results section only results of the ALK-1 antibody are given (Figure 1). Does this mean that the ALK-1 antibody was used to stain the 103 cases? What was then the purpose of the ALK11 antibody? In addition, dilutions of the ALK antibodies are missing in the methods section, while for all other antibodies dilutions are given.

Since ALK11 is a polyclonal antibody generated by Morris’ lab and more sensitive than ALK1, a monoclonal antibody which is less sensitive and expensive, so we initially assessed ALK expression by using the rabbit polyclonal antibody ALK11 (a kind gift from Dr. Stephan W. Morris, St. Jude Children’s Research Hospital) and further confirmed by using the mouse monoclonal antibody ALK-1 (Dako Cytomation, Carpinteria, CA) to exclude false positivity (ALK1 is less sensitive, but more specific than ALK11). Dilutions of the ALK antibodies have been added in the methods.

4. Methods; immunohistochemical studies: it is unclear how the immunohistochemical stainings were scored/interpreted. It is important to clarify this, since all study results are based on these stainings and IHC results are subject to interpretation. Are sections scored based on staining intensity levels?
Was the number of positive cells included / was there a cut-off for % of positive cells? When was a sample considered to be positive (for instance, if very weak staining was observed, was this considered to be positive or negative?)?

Who/how many persons were involved in interpreting the stainings? It is especially important to address these issues because the authors subsequently divide the cohort in “+ or –” expression for all the proteins they investigated, but IHC results are often not that black-and-white. In addition, representative pictures of both + and – scored slides for at least ALK, p-AKT and p-mTOR proteins should be included, and preferably also for p-4E-BP1 and p-p70S6K1.

Evaluation of the immunohistochemical staining was performed in a blinded set up regarding the clinical data. Scoring of the expression was performed semiquantitatively. In brief, both percentage of stained cells and staining intensity were evaluated. No staining or weak staining in <10% of cells was defined as 0, weak staining in at least 10% as 1, moderate staining in up to 50% as 2 and moderate staining in >50% of cells and strong staining of any percentage of the cells as 3.

The slides were read by at least three persons.

Since there were 6 positive IHC staining pictures of ALK, p-AKT, p-mTOR, p-4E-BP1, and p-p70S6K1, it would make the figure too big with the negative controls, so I omitted the all negative controls in the figure.

5. Results; The prognostic significance of the expression of ALK etc. section;

second paragraph: table VI is missing. In addition, VI should be 6 (in line with other tables).

In order to cut unnecessary tables and figures, I discard table 1. There are 4 tables in total. There is no table in the section of “The prognostic significance of the expression of ALK…”.

Minor Essential Revisions:

1. Abstract; methods: ATK/mTOR -># AKT/mTOR

Done!
2. Abstract; methods; “Expression of ALK and AKT/mTOR signaling phosphoproteins..” This sentence implies that the authors also looked at phosphorylated ALK protein, while only for AKT/mTOR phosphoantibodies were used. The sentence should be rewritten to avoid misinterpretation by readers.

I have rewritten the sentence as “Expression of ALK fusion proteins and the AKT/mTOR signaling phosphoproteins was studied by immunohistochemical staining.

3. Methods; study design: The 103 cases consist of 62 ALK+ALCL and 51 ALKALCL cases (gives total of 113 patients instead of 103)? Probably a mistake; adding up the ALK- cases in table 1 gives a total of 41 ALK- patients.

Yes, it was a mistake. I have made the correction.

4. Results: The prognostic..and p-p70S6K1; 1st paragraph: ALK, p-AKT, and p-mTOR signaling phosphoproteins -># ALK, p-AKT, and p-mTOR signaling proteins (p-AKT/p-mTOR already indicates phosphorylation)

I have made the correction.

5. Discussion: Information given in the first paragraph seems more appropriate for the introduction.

You are right! In this revised version, I placed the sentence “So far, at least 15 variant-ALK fusion genes have been found in both hematopoietic malignancies, such as ALCL and diffuse large B cell lymphoma, and non-hematopoietic neoplasms, including inflammatory myofibroblastic tumor, esophagus cancer, and non-small cell lung cancer” from Discussion to Introduction.

6. Discussion; 1st paragraph: 15 NPM-ALK variant fusion genes -> # 15 ALK-variant fusion genes?

I have made the correction.

7. Discussion; 3rd paragraph: kinsase -># kinase

I have made the correction.

8. Discussion; 3rd paragraph: AKT+/mTOR pathway -># AKT/mTOR pathway?

I have made the correction.
9. Discussion; 4th paragraph: prognositic -># prognostic

I have made the correction.

10. Figure 1 looks somewhat chaotic and confusing because many different magnifications are used. It feels more appropriate to show all pictures with the same, not too high, magnification (e.g. 200x), especially because remarks are made concerning the size of cells (Figure 1a shows large tumor cells and 1b small tumor cells, which is not surprising since 1a is 400x and figure 1b 200x). If the authors would like to highlight a specific staining pattern, I would suggest to include a higher inset magnification in addition to the lower magnification. The pictures are also a bit blurry; I would suggest to take clearer pictures which will make the manuscript look more professional.

Great suggestion! we have re-made the figure in which I used the clearer pictures and the same magnification of 400X.

11. Figure 1; end of legend: ‘No negative control staining was included’. This sentence suggests that no negative control stainings were performed at all, while in the methods section it is mentioned that controls were performed by omitting the primary antibody. If negative controls were used to verify the reliability of all antibodies used, and were indeed all negative, this sentence can be excluded from the figure legend to avoid misinterpretation.

You are right! I wanted to indicate that I omitted all of the negative control pictures in the figure.

Discretionary Revisions

1. Title: The authors state that the NPM-ALK/AKT/mTOR pathway is activated in ALCL. This however does not accurately convey what has been found. The authors have indeed found a correlation between ALK expression in general and p-AKT/p-mTOR, but they also state that neither p-AKT nor p-mTOR was related
to ALK subcellular expression patterns. Since these subcellular expression patterns distinguish NPM-ALK from other ALK-variants, they conclude that ‘no matter what the ALK fusions they are, they can activate AKT/mTOR pathway’ (language corrections also necessary in this sentence). The title suggests that specifically the NPM-ALK fusion correlates to AKT/mTOR pathway activation, but it could actually be any fusion. I would therefore suggest to delete the word ‘nucleophosmin’ from the title.

Great suggestion! I have deleted the word ‘nucleophosmin’ from the title.

2. Figure 1; legend: for all IHC stainings, ‘EnVision staining’ is mentioned, but this system is not specifically mentioned in the methods section. It would be logical to mention EnVision already in the Material and Methods section.

I have added the sentence “Immunohistochemical staining was performed to assess protein expression in formalin-fixed, paraffin embedded samples by the 2-step Envision procedure using a DAKO Autostainer (Dakopatts, Copenhagen, Denmark).” In the Methods section.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

Yes, the manuscript has been extensively edited.

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests
Reviewer 2's report

Title: Activation of nucleophosmin-anaplastic lymphoma kinase/protein kinase B/mammalian target of rapamycin signaling pathway in anaplastic large cell lymphoma and its correlation with the clinicopathologic variables

Version: 2 Date: 31 March 2013

Reviewer: Robert Roskoski

This is a very nice and important paper on signal transduction by protein kinase B and mTOR that fills an important gap in our knowledge.

Minor essential revision

On page 9 we are given the ages of patients as 25.32 +/- 15.25 where 25 +/- 15 would do. Similarly, 46.01 +/- 17.39 should be 46 +/- 17.

Yes! Thanks.

Level of interest: An article of outstanding merit and interest in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.
Reviewer 3's report

Title: Activation of nucleophosmin-anaplastic lymphoma kinase/protein kinase B/mammalian target of rapamycin signaling pathway in anaplastic large cell lymphoma and its correlation with the clinicopathologic variables

Version: 2 Date: 1 April 2013

Reviewer: Lukas Kenner

Reviewer's report:

The manuscript Ju Gao et al. „Activation of nucleophosmin-anaplastic lymphoma kinase/protein kinase B/mammalian target of rapamycin signaling pathway in anaplastic large cell lymphoma and its correlation with the clinicopathologic variables“ show that in ALK-negative samples are overrepresented in p-AKT negative samples, suggesting an relative higher activation of the AKT pathway in the patients with ALK translocations.

This is an interesting manuscript, however a number of the data shown were already published elsewhere. In addition the study completely relies on IHC staining of patient samples and do not include any more mechanistic in vivo data to support the authors hypotheses, which would be important to support the translational relevance of this manuscript for possible future diagnostic and therapeutic approaches.

In addition, the authors did not verify their data with state of the art quantification techniques.

Major:

As expected, the mTOR pathway is mostly higher activated in patients bearing the ALK translocation. However localization of the NPM-ALK fusion protein seems to be of no consequence for prognosis and pathway activation in ALCL.
Therefore the data suggest that the AKT-mTOR axis is over-activated in 70-80% ALK+ ALCL cases. This suggests that mTOR as well as the AKT pathway are crucial for tumor growth in ALCL, ALK+ and probably less so in ALCL, ALK-, however this is not proven in the current version of the manuscript.

1. the authors should use ALCL cell lines (Karpas 299, SR786, SU-DHL1, Mac1, Mac2a) with and without the ALK translocation and treat them with different AKT and mTOR inhibitors, such as NVP-BEZ235, which is a dual PI3K and mTOR inhibitor.

This would give direct proof about the importance of the described pathways for ALCL tumor growth in ALK+ and ALK- tumors.

Since the AKT1 kinase has two homologs AKT2 and AKT3, it would be interesting to know the respective contributions of these homologs to the AKT/mTOR pathway activation. This should be feasible since there are inhibitors available that bear a certain specificity for the different AKT homologs.

Alternatively, the authors could do independent knockdown of AKT1/2/3 using antisense or small hairpin or siRNA techniques in the cell lines mentioned above and study activity of the AKT/mTOR pathway by 4EBP1 and S6K phosphorylation to dissect the differential importance of the homologs.

Yes, in this revised manuscript we add some mechanistic study. We overexpressed NPM-ALK in a nonmalignant murine pro-B lymphoid cell line, BaF3, which induced the cells to become cytokine-independent growth and resistance to glucocorticoids. We demonstrated that a more hyper-activated AKT/mTOR pathway in BaF3 cells stably transfected by NPM-ALK than in BaF3 cells transfected with empty vector or the parental BaF3 cells and targeting AKT/mTOR inhibited growth and triggered the apoptotic cell death of ALK+ ALCL cells and NPM-ALK transformed BaF3 cells, and also reversed the drug resistance induced by overexpression of NPM-ALK.

We are doing now with ALK+ ALCL cell lines and BaF3/X-ALK cells to address which of the downstream effectors, AKT1, AKT2, AKT3, mTORc1 and mTORC2, plays a role in NPM-ALK’ transforming activity. We will publish these data in another paper.
2. To demonstrate the in vivo significance of their findings, authors should xenograft the above mentioned cell lines into mice and demonstrate the growth pattern and target gene expression levels after mTOR and AKT1/2/3 inhibition.

Yes, Great suggestion. We will do it in the future.

3. State of the art quantification software (such as Histoquest or else) should be applied to quantify protein expression levels and percentage of tumor/stromal cells affected from the tumor samples.

Great suggestions!

Our slides were read by at least three persons.

Evaluation of the immunohistochemical staining was performed in a blinded set up regarding the clinical data. Scoring of the expression was performed semiquantitatively. In brief, both percentage of stained cells and staining intensity were evaluated. No staining or weak staining in <10% of cells was defined as 0, weak staining in at least 10% as 1, moderate staining in up to 50% as 2 and moderate staining in >50% of cells and strong staining of any percentage of the cells as 3.

Minor:

1. On page 8 a table VI is mentioned, which should be shown.
   No table 6. I have changed the sentence.

2. Please carefully correct several typos.

Yes, I did. I have highlighted all the corrections in the manuscript.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being Published

I have revised the manuscript again and made all the corrections I have found and you indicated above. Also I asked a native-English speaker who is professional in molecular pathology to help me with language corrections. I have highlighted all the corrections in the manuscript.
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

I declare that I have no competing interests