Author's response to reviews

Title: MTDH Mediates Trastuzumab Resistance in HER2 Positive Breast Cancer by Decreasing PTEN Expression through an NF-kappa B dependent Pathway.

Authors:

Cheng du (dc1115010@sina.com)
Xiaomin yi (yiserpent163@163.com)
Wenchao liu (xicancer@fmmu.edu.cn)
Tao han (doctor_than@163.com)
Zhaozhe liu (doctor_lzz@163.com)
Zhenyu ding (doctor_dzy@163.com)
Zhendong zheng (doctor_zzdong@163.com)
Ying piao (doctor_py@163.com)
Jianlin yuan (yuanjianlin317@126.com)
Xiaodong xie (doctor_xxd@163.com)
Yaling han (hylcardiology@163.com)
Manjiang xie (xiemanjiang@163.com)

Version: 4
Date: 26 October 2014

Author's response to reviews: see over
Oct 25th, 2014  

Dr. Ryan M. Relox  
Editor, *BMC Cancer*  

**RE:** Manuscript version 1 entitled “MTDH Mediates Trastuzumab Resistance in HER2 Positive Breast Cancer by Decreasing PTEN Expression through an NF-kappa B dependent Pathway.” MS: 1422717538135513.  

Dear Dr. Ryan M. Relox,  

Thank you very much for your letter of Sep 26, 2014 with regard to the review of the above-mentioned manuscript for consideration for publication in *BMC Cancer*. We are very grateful to the reviewers’ positive comments. We really appreciate the encouragement from both reviewers and the editor.  

We also thank the reviewers for the thorough critiques that are very helpful in preparing this revised submission. We have carefully considered each of the comments from two reviewers, performed extra experiments, and made changes to the manuscript accordingly. Below are our point-to-point responses, in which each comment from the reviewers is highlighted in grey. The revised texts in manuscript are highlighted in red.  

**Minor essential revision.**  

1. Results, paragraph “Forced expression in SKBR-3/r cells restored trastuzumab sensitivity.” When they say “Furthermore, SKBR-3 cells with GFP-PTEN, should say...”
Response: We apologize for this. Thanks for the careful reviewer’s comments on our manuscript. This has been corrected.

2. In legend of Figure 2A should be indicated the concentration of Trastuzumab used.
Response: Great thanks for the reviewer’s advice. The concentration of Trastuzumab has been indicated according to reviewer’s advice as follows.
A. Relative proliferation of SK-BR-3 and SK-BR-3/R cells under trastuzumab exposure (5μg/ml) at different time points by MTT assay (*P<0.05).

3. In legend of Figure 3B should be indicated the duration of the trastuzumab treatment and the concentration used.
Response: Thanks for the comments. The concentration and duration of Trastuzumab has been indicated as follows.
B. MTT assay indicated that MTDH-shRNA significantly reduced trastuzumab resistance in SK-BR-3/R cells, while MTDH up-regulation led to the resistance of trastuzumab (5μg/ml, 72h) in SK-BR-3 cells (*P<0.05).

4. In figure 3B, 3C and 3D should be indicated that knockdown is down in SKBR-3/R cells and overexpression in SKBR-3.
Response: Thanks for the comments. This has been mentioned in figure legend as follows.
MTDH was overexpressed in SK-BR-3 cells and knocked down in SK-BR-3/R cells.

5. In figure S1 should be indicated that knockdown is down in SKBR-3/R cells and overexpression in SKBR-3.

Response: Thanks for the comments. This has been indicated in figure S1 as follows.

Figure S1. Representative images of 5-ethyl-2′-deoxyuridine (EdU) incorporation assay. MTDH was overexpressed in SK-BR-3 cells and knocked down in SK-BR-3/R cells.

6. In figure S2C, correct X axis.

Response: We apologize for this. The figure has been corrected.

Major compulsory revision

1. In figure 4C: The knockdown of p65 or MTDH has a similar effect in the relative
levels of PTEN luc activity and the knockdown of both proteins have an additive effect, do the authors have check the effect of an NFκB inhibitors in this resistant cell line in the levels of PTEN and if this treatment restore the response to trastuzumab.

Response: Thanks for the precious suggestion from the reviewer. We demonstrated that knockdown of both proteins p65 and MTDH have an additive effect. When NF-kB inhibitor BAY11-7082 (10μmol/L) was applied, a similar effect on PTEN-luc activity in the resistant cell line was observed. PTEN expression was also restored. We also found that BAY11-7082 treatment partly restore trastuzumab sensitivity in this resistant cell line. This was in consistent with previous studies (Dong X et al., 2013, J Cell Biochem; Kim S et al., 2004, J Biol Chem; Chow JY et al., 2010, Am J Physiol Gastrointest Liver Physiol).

2. In figure 5A: the authors show the expression of endogenous PTEN and PTEN-GFP.
they claim that they see an increase in PTEN levels, but the size of PTEN and PTEN-GFP is different, the band that they show in the picture correspond to PTEN or PTEN-GFP? It could be better if the show in the same picture both the endogenous and PTEN-GFP.

Response: This is an important question. We totally agree with the reviewer’s comment that the size of PTEN (54kDa) and PTEN-GFP (71kDa) fusion protein is different. In most gene expressing systems, GFP (or eGFP) and the target gene are expressed as a fusion protein through one promoter. In other cases, however, they can be expressed simultaneously and separately if the vector contains an IRES sequence. In our study, we used an expressing vector containing such structures (as shown below). GFP is promoted by an internal ribosome entry site (IRES) in the vector. The protein expressed by the vector is not a fusion protein of PTEN-GFP. Instead, PTEN and GFP are separately expressed. Therefore, the molecular weight of exogenous PTEN is similar with that of endogeneous PTEN (54kDa).
3. Also in figure 5A there is not a complete downregulation of AKT in those cells that overexpress PTEN, do the authors could explain that. If they treat SKBR3/R cells with a PI3K inhibitor do they see a complete inhibition of AKT?

Response: We appreciated this valuable question. The transfection efficiency of PTEN-GFP plasmid maintains around 70% using traditional transfection method described in the manuscript. We figured that remnant cells might affect the complete downregulation of pAkt since western blot analysis included all these cells. To further test the effect of PI3K inhibitor on the phosphorylation of AKT in SK-BR-3/R cells, we used a PI3K inhibitor LY-294002 (50μmol/L) in combination with trastuzumab for the treatment of SK-BR-3/R cells and found that phosphorylation of AKT was almost completely inhibited (as shown below). These results are in consistent with previous studies (Liu et al., 2011, Cell Cycle; Cheng et al., 2009, Cancer Letters).

4. In figure 5C: overexpression of PTEN-GFP clearly diminish the proliferation capacity of the cells but even though statistically significant when they treat this cells with trastuzumab they don’t see a strong response. Do the authors have any explanation for this result?

Response: Thanks for the reviewer’s question. I guess that the reviewer asked the
Figure 5B (proliferation) but not 5C (apoptosis). As shown in Figure 5B, when PTEN was overexpressed in SK-BR-3/R cells, the relative proliferation was 54% of the control group (100%). When trastuzumab was added, the relative proliferation was 34% of the control group (100%). It can be calculated that trastuzumab inhibited the proliferation of PTEN overexpressed SK-BR-3/R cells by 37%. Given that most targeted therapeutics exert effects in a relative moderate manner compared with chemotherapeutics, this number (37%) may be interpreted as a good response, although it seems from the figure that the response is not so strong. Furthermore, it is possible that PTEN overexpression and trastuzumab treatment may partially share similar downstream mechanism in suppressing the proliferation of SK-BR-3 cells, which makes the additive effect seem not so strong.

5. As the authors claim that MTDH upregulation in resistant cell lines regulates the levels of PTEN inducing an activation of AKT, do this resistant cell line respond to PI3K inhibition and combination with trastuzumab restore its sensitivity. As this combination treatments are already in the clinics and it would be important to see that those resistant tumors with high levels of MTDH could also respond to the combination treatment with PI3K inhibitors and trastuzumab.

Response: We really appreciated this valuable question and we examined whether SK-BR-3/R cells respond to the combination treatment with PI3K inhibitors and trastuzumab. As previously mentioned in our response to Q#3, PI3K inhibitor LY-294002 (50μmol/L) significantly inhibited phosphorylation of Akt in the resistant
cells. Combined treatment of LY-294002 and trastuzumab could restore its sensitivity (as shown below). This is consistent with previous preclinical studies (Liu et al., 2011, Cell Cycle; Cheng et al., 2009, Cancer Letters; Chakrabarty A et al, 2013, Cancer Res; Rexer BN et al, 2014, Breast Cancer Res). Moreover, the combination treatment with trastuzumab and inhibitors for PI3K/Akt signaling pathway has also been proved to be effective in several clinical studies (André F, 2014, Lancet Oncol; Hudis C, 2013, Breast Cancer Res). Therefore, it would be of great importance to investigate whether trastuzumab resistant tumors with high levels of MTDH could also respond to this combination treatment.

![Graph showing cell viability](image)

6. In figure 6C: IHC for MTDH should be included to show that knockdown and overexpression of MTDH has been kept along the treatment.

Response: We totally agree with the reviewer about this point. We have included IHC for MTDH in figure 6C.

Response: Thanks to the reviewer’s question. This reference and our comments have been included in the manuscript.

MTDH is established as an oncogene and related with proliferation, angiogenesis and metastasis in cancer cells through participating in multiple oncogenic pathways such as Ha-Ras, myc, NFkB, and PI3K/Akt. In addition, MTDH is also involved in drug resistance to chemotherapeutics and tamoxifen. The multiple functions of MTDH in drug resistance highlight that it may serve as a viable target for a variety of cancers. Due to the lack of large perspective clinical studies, however, it is still difficult to
determine whether MTDH is a general or a specific mechanism of drug resistance in certain cancers. Based on the preclinical studies of MTDH in breast cancer, it seems that MTDH may serve as a general mechanism for drug resistance. However, more work needs to be done to expand its value, especially the validation in large randomized clinical studies.

As far as we know, two studies (Ward A, 2013, Oncogene; Xu C, 2014, Cell Physiol Biochem) have reported that MTDH may participated in tamoxifen resistance. Ward et al. identified MTDH as a direct target of miR-375 in breast cancer. Knockdown of MTDH partially phenocopied the effects of miR-375 on the sensitivity to tamoxifen in in-vitro models. They also observed that adjuvant-tamoxifen-treated patients with higher expression of MTDH had a shorter disease-free survival and higher risk of relapse. They concluded that re-expression of miR-375 or targeting MTDH might serve as potential therapeutic approaches for the treatment of TamR breast cancer. However, the mechanism by which MTDH mediates tamoxifen was not investigated in the above mentioned study. Moreover, the conclusion needs to be further validated in in-vivo models and in the context of advanced tamoxifen resistance breast cancer patients.

Xu et al. recently reported that overexpression of MDTH mediated estrogen-independent growth and induced resistance to tamoxifen in in ERα-positive MCF-7 breast cancer cells. They also found that MTDH reduced the expression of PTEN, up-regulate AKT and BCL2 and inhibit the apoptosis induced by tamoxifen. In consistent with these findings, our study also demonstrated that MTDH negatively regulated PTEN. Although we used a different cell line (ER negative and HER2 positive), there is no contradiction between these two studies, because PTEN is reported to be involved in both tamoxifen-resistance (Tanic N, 2012, Cancer Biol Ther; Lindberg K, 2011, Breast Cancer Res. Phuong NT, 2011, Breast Cancer Res Treat) and trastuzumab-resistance (as mentioned in the manuscript) in breast cancers. However, the findings from Xu’s study need to be validated in in-vivo models and
require to be investigated in clinical samples. In addition, the potential mechanism of interconnection and crosstalk between MTDH and PTEN should also be probed. Taken together, whether and how MTDH mediates drug resistance in certain molecular subtype of breast tumors needs to be mechanistically investigated and clinically validated in the future.

In summary, we sincerely appreciate the reviewers’ helpful comments. Accordingly we performed more experiments in last month and made substantial changes in the revised manuscript as outlined above. The new evidence supports our hypothesis that MTDH mediates trastuzumab resistance in HER2 positive breast cancer by decreasing PTEN expression through an NF-kappa B dependent pathway. We hope that both reviewers will show stronger enthusiasms and accept this revised manuscript for publication in BMC Cancer.