Author's response to reviews

Title: The association of TP53 mutations with the resistance of colorectal carcinoma to the insulin-like growth factor-1 receptor inhibitor picropodophyllin

Authors:

Quan Wang (wangquan-jlcc@hotmail.com)
Feng Wei (weifeng78@hotmail.com)
Guoyue Lv (lgy08@sina.com)
Chunsheng Li (shenglong1226@163.com)
Tongjun Liu (tongjunliu@163.com)
Guikai Zhang (gzhang4@emory.edu)
Anita Bellail (abellai@emory.edu)
Chunhai Hao (chao@emory.edu)

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Author's response to reviews: see over
RE: MS: 1378166039976762

“The association of TP53 mutations with the resistance of colorectal carcinoma to the insulin-like growth factor-1 receptor inhibitor picropodophyllin”

Dear Dr Reid

First, please accept my apology for the 3 days delay in resubmission in part due the extra time unexpected in completion of the experiments. Most importantly, we are grateful for your kindly providing the opportunity of resubmission after major revision.

We equally appreciate the constructive comments from the two reviewers: Prof Leonard Girnita and Prof Jin-Ming Yang, both are renowned and well-respected researchers in the fields. We have therefore made the best effort to address all the comments in the revision.

The point-by-point response (R) to the comments in italic (C) follows:

Reviewer, Prof Leonard Girnita’s comments:

Major Compulsory Revisions

As explained in the introduction, PPP was originally discovered as an IGF-1R kinase inhibitor. However, subsequent studies demonstrated the besides inhibiting IGF-1R phosphorylation, PPP also induces IGF-1R downregulation through a mechanism involving the receptor ubiquitination (PMID: 17828296). As long as p53 and IGF-1R share the same ubiquitin ligase MDM2 (PMID: 12821780) and this ligase is under the transcriptional control of the wt p53, the results should be also analyzed and interpreted in this context.

C 1. One possibility is that different expression of MDM2 among different colon carcinoma cell lines, modify the PPP induced IGF-1R downregulation, thus explaining their higher sensitivity to PPP. To get support for this mechanism I would suggest investigating the expression status of MDM2 as well as the kinetic of IGF-1R degradation (0, 12 and 24 h) following treatment with IGF-1, PPP and PPP+IGF-1. Increased (or decreased through receptor recycling) receptor degradation in wtp53 versus mtp53 cell lines might provide clues regarding the mechanism of their sensitivy.
R1: Following the guidance of Prof Girnita, we have carried out these two experiments with the data presented in Figure 2B and Figure 2C. The text was revised with the references cited as follow (page 7, last paragraph):

“Earlier studies have clearly shown that PPP treatment leads to the downregulation of IGF-1R through MDM2-mediated ubiquitination and degradation of the IGF-1R protein [35]. Both IGF-1R and p53 proteins are the substrates of the ubiquitin ligase MDM2 [36]. To explore the role of MDM2 in the resistance of mutated TP53 cell lines to PPP, we examined the protein levels of MDM2 in wild and mutated TP53 cell lines by western blotting. The data revealed no difference in the expression of MDM2 protein between TP53 wild type and mutated cell lines (Figure 2B). Next, we examined the kinetic of IGF-1R degradation under the treatment of IGF-1 and PPP, alone and in combination. To this end, we compared the IGF-1R protein levels between the TP53 wild type SW948 and mutated CACO-2 because these two cell lines expressed IGF-1R protein at similar levels (Figure 1B). Western blotting revealed that PPP treatment reduced the levels of IGF-1R protein in both SW948 and CACO-2 cells (Figure 2C) due to the similar expression levels of MDM2 protein between these two cell lines (Figure 2B). These results confirm the earlier reports [35, 36] that PPP treatment induces the IGF-1R degradation through MDM2-mediated ubiquitination in a p53 independent manner.

C 2. An apparent paradox is presented in Fig. 2A for the CACO-2 cell line: although the kinase activity of the IGF-1R is inhibited by PPP (as demonstrated by receptor phosphorylation) the AKT and ERK are (hyper) phosphorylated. Thus, another possibility that should be taken in consideration to explain the limited response to PPP in cells expressing mtp53 is the “biased” signaling activation by PPP. As described above, what differentiate PPP from other IGF-1R tyrosine kinase inhibitors is receptor down-regulation. On the other hand the IGF-1R down-regulation is a common feature for anti-IGF-1R targeting antibodies. It has been shown that PPP induced receptor down-regulation activates ERK signaling (PMID: 18070930). Likewise, in the case of anti-IGF-1R antibodies, it has been demonstrated that “biased” IGF-1R biased signaling is responsible for resistance to anti-IGF-1R therapy (PMID: 23188799). Therefore I would suggest testing the kinetic of ERK phosphorylation following short time (0 to 60 min) stimulation with PPP only in serum-free media.

R 2. Yes, we have carried out the experiment as Prof Girnita suggested with the data presented in Figure 2D) and manuscript revised as follow (page 8, the first paragraph):

“The studies from the same research team have further demonstrated that the MDM2-mediated ubiquitination of IGF-1R under PPP treatment leads to the activation of ERK pathway [37], resulting in the resistance of Ewing’s sarcoma to the treatment of the anti-IGF-1R antibody figitumuab [38]. To explore this mechanism in colorectal carcinoma, we treated SW948 and CACO-2 cell lines with PPP in a dose dependent manner and showed that PPP treatment increased the levels of p-ERK in the TP53 mutated CACO-2 but not in the TP53 wild type SW948 cells (Figure 2D). Taken together, the results suggest that PPP treatment blocks the phosphorylation of IGF-1R and inhibits the downstream ERK pathway in TP53 wild type
colorectal carcinoma cells. In contrast, TP53 mutated carcinoma cells are resistant to the PPP treatment in part due to its failure of inhibition of intracellular ERK pathway.”

C 3. Nevertheless the possible resistance mechanisms to PPP as well as demonstrated signaling activation with low IGF-1R phosphorylation in mtp53 versus wtp53 mtp53 should be addressed in the discussion.

R 3. Yes, we have added a paragraph in the discussion as follow (page 11, last paragraph):

“A research group at the Karolinska Institute has made the discovery of PPP as an IGF-1R inhibitor [25] and revealed the mechanism of the action that PPP blocks the phosphorylation of IGF-1R [26] and induces G2/M-phase accumulation and apoptosis [27]. This group has further shown that PPP treatment down-regulates IGF-1R protein through MDM2-mediated ubiquitination and degradation [35] and the MDM2-mediated IGF-1R ubiquitination activates ERK pathway [37] and leads to the cancer resistance to PPP [38]. The data presented in this manuscript have confirmed the action of PPP in inhibition of cell growth and induction of apoptosis in TP53 wild type colorectal carcinoma cells. On the other hand, the data suggest the correlation between TP53 mutation and PPP resistance in the carcinoma cells. Both p53 and IGF-1R proteins are the substrates of MDM2; however, the presence of MDM2 in both TP53 wild type and mutated carcinoma cells suggests that PPP-induced ERK activation in TP53 mutated carcinoma cells occurs through a p53-independent manner. The PPP-induced ERK activation contributes in part to the resistance of TP53 mutated colorectal carcinoma to the IGF-1R inhibitor PPP.”

Minor Essential Revisions

C 4. There are some spelling errors or missing words. For the PPP description I would suggest using the term “IGF-1R inhibitor” instead “IGF-1R kinase specific inhibitor”.

R 4. Yes, we have corrected spelling errors, added missing words and revised “IGF-1R kinase specific inhibitor” to “IGF-1R inhibitor” through the manuscript.

Reviewer, Prof Jin-Ming Yang’s comments:

Minor points:

C 1. Fig. 1C and Fig. 4A (Fig. 5A in revision): is there only one point that is statistically significant?

R 1. In the revision, we have made it clear that the experiment was repeated three times, the data were analyzed statistically and the significant difference was indicated in the figures.
C 2. Fig. 2C (Fig. 3B in revision): Statistical analysis should be performed.

R 2. Following the suggestion, we have performed the statistical analysis and the significant difference was indicated in the figure.

C 3. Carful proofreading is required.

R 3. Yes, we have carried out a carful proofreading and revised any mistakes.

We feel that the manuscript has been improved significantly after the revision and hope that the revised manuscript is suitable for publication in your journal.

Sincerely yours,

Chunhai “Charlie” Hao, MD, PhD, FRCPC