Peer Review File

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Comments to Reviewers

1. The authors should perform MTHFD2 overexpression in HCC78 cells, the lowest expression level of MTHFD2, to demonstrate the Pemetrexed resistance function of MTHFD2.

First, we want to thank you much for your time and efforts reviewing our manuscript. We are grateful for your comments, which we believe will improve our current manuscript.

We agree with the reviewer that the correlation between MTHFD2 expression and resistance to treatment with Pemetrexed is an important point also for future clinical decisions of treatment selection for patients with pulmonary adenocarcinoma. To further investigate and underpin our finding that strong MTHFD2 expression leads to treatment resistance against Pemetrexed we have approached this issue from two sides.

First, as requested, we transfected the cell line HCC78 with a human MTHFD2 overexpression plasmid or an empty vector plasmid as control. However, even after testing several different conditions of transfection, the cells did not survive the transfection. Therefore we have overexpressed MTHFD2 in the H1993 cell line, which also presents low MTHFD2 expression and good response to Pemetrexed. Cell viability assay showed that overexpression of MTHFD2 in H1993 cells markedly increased the IC50 of cells after treatment with increasing concentrations of Pemetrexed compared to empty vector control cells (IC50 for Pemetrexed: 0.30 μM and 0.081 μM, respectively, Figure 7E).

Secondly, we knocked down MTHFD2 in HCC44 that virtually do not respond to treatment with Pemetrexed and presented the highest level of MTHFD2 protein expression. Cell viability assay revealed that MTHFD2 silencing significantly reduced viability of cells after treatment with increasing concentrations of Pemetrexed compared HCC44 cells transfected with control siRNA (IC50 Pemetrexed: 30.2 μM and 110.7 μM, respectively, Figure 7F). This indicates that the sensitivity to Pemetrexed was restored after knockdown of MTHFD2 in HCC44 cells.

Changes in the text:

1) Page 7, line 135-139, highlight in yellow: "Either a pcDNA3-MTHFD2 vector (CloneID: OHu18706, GenScript) or an empty pCDNA3 vector backbone (138209, Addgene) was transfected into AC cell lines for overexpression of MTHFD2. Cells expressing de-novo MTHFD2 were selected with G418 (at concentration of 700μg/mL) for at least one week and MTHFD2 protein levels were confirmed by Western Blotting"

2) Page 12, line 245-255, highlight in yellow: "To explore the Pemetrexed resistance function of MTHFD2 in AC cells, we transfected a pcDNA-MTHFD2 or an empty vector or a specific siRNA against MTHFD2 or a non-target control siRNA in AC cells and treated the resulting cells with increasing concentrations of Pemetrexed. Overexpression of MTHFD2 in H1993 cells markedly increased viability of cells when treated with Pemetrexed compared to empty vector treated cells (IC50 for Pemetrexed: 0.30 µM and 0.081 µM, respectively, Fig. 7E, Suppl. Fig. 8A+B). In contrast, MTHFD2 silencing significantly reduced cell viability after Pemetrexed treatment when compared to control treatment in HCC44 cells (IC50 Pemetrexed: 30.2 µM and 110.7 µM, respectively, Fig. 7F). This indicated that resistance to Pemetrexed was induced by MTHFD2 overexpression and restored by its knockdown in AC cells."

3) Page 14, line 294-297, highlight in yellow: "We furtherly confirmed the correlation between MTHFD2 expression and resistance to Pemetrexed by showing that overexpression of MTHFD2 leads to increased resistance to Pemetrexed meanwhile knockdown of MTHFD2 reduces resistance to Pemetrexed."

4) We have revised Figure 7 and the legend of Fig. 7 (Page 22, line 499-506, highlight in yellow) as follows: "IC50 values and inhibitory curve in each cell line after treatment with Pemetrexed tested in lung cancer cell lines of AC (A) and SCLC (B). Scatterplots of relative MTHFD2 expression vs IC50 values of Pemetrexed in 5 AC (C) and 5 SCLC (D) cell lines. Pearson correlation coefficient (r) and P value are displayed. Representative results of cell viability assay after treatment with Pemetrexed in MTHFD2-overexpressing H1993 cells, empty vector transfected H1993 cells (E), MTHFD2-knocked down HCC44 cells and negative control siRNA transfected HCC44 cells (F). Data is represented as mean ± SEM of three independent experiments."

5) Furthermore, we have added a new supplementary Figure 8 and a corresponding figure legend (Page 24, line 551-554, highlight in yellow): "**Supplementary Figure 8: Overexpression of MTHFD2 in vitro H1993 cells.** (A) H1993 cells were stably transfected with a human MTHFD2 gene expression vector or an empty vector (EV). (B) Bar chart showed quantification of protein levels

compared to PARK7 control. Data is represented as mean ± SEM of three independent experiments."

2. The legends of Figure 7 are required to be corrected. Fig. 7D should be the correlation data of SCLC cells, not AC cells.

We appreciate that the Reviewer has pointed out this typographic error. We have revised the legends of Figure 7 in the Legends section accordingly.

Changes in the text:

Page 22, line 499-506, highlighted in yellow: "IC50 values and inhibitory curve in each cell line after treatment with Pemetrexed tested in lung cancer cell lines of AC (A) and SCLC (B). Scatterplots of relative MTHFD2 expression vs IC50 values of Pemetrexed in 5 AC (C) and 5 SCLC (D) cell lines. Pearson correlation coefficient (r) and P value are displayed. Representative results of cell viability assay after treatment with Pemetrexed in MTHFD2-overexpressing H1993 cells, empty vector transfected H1993 cells (E), MTHFD2-knocked down HCC44 cells and negative control siRNA transfected HCC44 cells (F). Data is represented as mean ± SEM of three independent experiments."

3. The knockdown of MTHFD1 displayed a similar growth inhibition in AC cells to MTHFD2 knockdown. Why the authors only focus on MTHFD2?

We appreciate the reviewer pointed out this important issue. MTHFD2 catalyzes the transformation of methylene tetrahydrofolate and is one of the major enzymes involved in mitochondrial folate one-carbon metabolism. Depletion of MTHFD2 could cause cell death in multiple cancers and impair key features associated with cancer progressions, such as proliferation, invasion, migration, and metastasis. (Ref 7, Ref 11, Ref 12, Ref 34, Ref 41).

Due to the following reasons in our current study we have focused on MTHFD2 instead of MTHFD1:

1. We did not find a significant correlation of MTHFD1 with patient survival in any of the tested groups (Suppl. Figure 1C)

2. MTHFD1 protein is expressed in all adult tissues (PMID: 2468308) but MTHFD2 is almost exclusively expressed in cancer cells and is absent in most adult tissues (Ref 11, PMID: 3877056), so we believe that MTHFD2 is a better candidate for a potential new drug target.

4. The expression level of TYMS among AC cells displayed a similar pattern to MTHFD2 (Fig. S2). It suggests that there should be a positive correlation between Pemetrexed resistance and TYMS expression level. The authors should also perform such analysis.

Again, we appreciate the reviewer's suggestion and have performed the correlative analyses with TYMS expression and Pemetrexed resistance and summarized this data in the Supplementary Figure 6. We also revised the Results section and the Discussion accordingly.

Changes in the text:

1) Page 12, line 241-244, highlight in yellow: "However, we did not observe any further significant correlation between 1CM proteins expression and drug sensitivity. There was a tendency for better response to Pemetrexed treatment in TYMS low expression AC and SCLC cells (r= 0.27, P = 0.06, and r= 0.84, P = 0.06, respectively, Suppl. Fig. 6A+B)."

2) Page 15, line 311-316 and Page 20, line 455-461, highlight in yellow: "Although the correlative analyses with TYMS expression and Pemetrexed resistance did not produce a statistically significant correlation, there was also a trend for better response to Pemetrexed treatment in TYMS low expression AC and SCLC cell lines (r= 0.27, *P* = 0.06 and r= 0.84, *P* = 0.07, respectively, Suppl. Fig. 6A+B). These findings support previous studies, which suggested that TYMS was a predictive factor for sensitivity to Pemetrexed in lung cancer (19,43,44)."

3) New references:

43. Christoph DC, Asuncion BR, Hassan B, et al. Significance of folate receptor alpha and thymidylate synthase protein expression in patients with non-small-cell lung cancer treated with Pemetrexed. J Thorac Oncol 2013;8:19-30.

44. Agullo-Ortuno MT, Garcia-Ruiz I, Diaz-Garcia CV, et al. Blood mRNA expression of REV3L and TYMS as potential predictive biomarkers from platinum-based chemotherapy plus Pemetrexed in non-small cell lung cancer patients. Cancer Chemother Pharmacol 2020;85:525-35.

5. The black-to-white labels of Figure 4 to 6 make them difficult to distinguish. The authors should consider changing them to color labels.

We agree and have adjusted the figure accordingly.

Changes in the text:

We have modified the color labels of Figure 4, 5, 6 and Suppl. Figure 4.

cklist for Authors" form accordingly.