Peer Review File

Article Information: https://dx.doi.org/10.21037/jgo-22-862

Reviewer A:

Study is limited by small numbers and hypothesis generating only regarding interaction between methylation and survival outcomes, but intriguing and warrants publication.

I think the authors need to include results of a multivariate analysis for PFS and OS that combines cofactors of RAS status/BRAF mutation status/ primary tumour side and methylation to see if methylation is an independent predictor for survival.

Reply: Thank you for your suggestion to add multivariate analysis for PFS and OS. Accordingly, we have added the results of univariate and multivariate analyses for PFS and OS (Table 4). The results did not indicate that the DNA methylation status was an independent predictor of PFS and OS. We believe that there are two reasons for this: the majority of HMCC patients had *RAS* or *BRAF* mutations, and the combined chemotherapy had an effect on the response to the treatment. DNA methylation status is known to be associated with *BRAF* and *RAS* gene mutations, and all cases with *BRAF* mutation (n = 8) and two cases with *RAS* mutations were classed as HMCC. Among a total of 15 HMCC cases, 67% (10 cases) were *RAS*/*BRAF* mutations, which may have eliminated the significance of DNA methylation status on PFS and OS. Furthermore, DNA methylation status has been reported to be associated with sensitivity to anti-EGFR antibodies, but not to chemotherapy. Therefore, in the case of HMCC tumors that are sensitive to chemotherapy, therapeutic benefit can be achieved from the chemotherapy with anti-EGFR antibodies. This may have attenuated the predictive power of DNA methylation status for the treatment response form anti-EGFR antibodies.

On the other hand, as shown in Figs. 3C and D, in *RAS*/*BRAF* wild-type patients, the mPFS and mOS in the LMCC group was numerically better than those in the HMCC group (ΔmPFS, 5.2 M; ΔmOS, 8.8 M), although the difference was not statistically significant. We believe that our hypothesis that DNA methylation status is an independent predictor of mPFS and OS needs to be validated in future larger trials.

We have added the following sentences.

Page 9, Line 218–219: “In uni- and multivariate analysis for PFS and OS, hazard ratios (HRs) and their CIs in the COX proportional hazards model were calculated.”

Page 12, Line 287–294: “Univariate analysis of PFS in all eligible cases showed that *RAS* gene mutation, *BRAF* gene mutation, and DNA methylation status were significantly associated with PFS (Table 4). Multivariate analysis of these three factors showed that *RAS* and *BRAF* mutations were significantly associated with PFS. Univariate analysis of OS showed that primary tumor resection, presence of poorly differentiated component, *BRAF* mutation, and DNA methylation status were significantly associated with OS, whereas multivariate analysis of these four factors showed that primary tumor resection and *BRAF* mutation were significantly associated with OS.”

Page 16, Line 379–393: “Results of the present study indicated that DNA methylation status was also significantly associated with PFS and OS in the univariate analysis, and HMCC group showed a significantly poorer treatment outcome than LMCC, but not in multivariate analysis. DNA methylation status is known to be associated with *BRAF* and *RAS* gene mutations, and all cases with *BRAF* mutation

(n = 8) and two cases with *RAS* mutations were classed as HMCC. Among a total of 15 HMCC cases, 67% (10 cases) were *RAS*/*BRAF* mutations, which may have eliminated the significance of DNA methylation status on PFS and OS. In addition, DNA methylation status has been reported to be associated with sensitivity to anti-EGFR antibodies, but not to chemotherapy. Therefore, in the case of HMCC tumors that are sensitive to chemotherapy, therapeutic benefit can be achieved from the chemotherapy with anti-EGFR antibodies. This may have attenuated the predictive power of DNA methylation status for the treatment response form anti-EGFR antibodies. Studies are required to examine the significance of DNA methylation status as predictors of treatment response to anti-EGFR antibodies in a larger number of patients without *RAS*/*BRAF* mutations."

Also suggest amending conclusion to say .
"DNA methylation status warrants further exploration as a predictive biomarker for anti-EGFR efficacy in metastatic colorectal cancer."

Reply: We agree with you and have modified the conclusion as per your suggestions (see Page 4, Line84–85. and Page 18, 430–431).

Would also suggest changing the title to: DNA methylation status as a predictable biomarker of cetuximab efficacy during second-line treatment of metastatic colorectal cancer: a T-CORE 1201study

Reply: We changed the title to the following as per Reviewer A and B's suggestion.
"Phase II study of biweekly cetuximab plus mFOLFOX6 or mFOLFIRI as second-line treatment for metastatic colorectal cancer and exploratory analysis of associations between DNA methylation status and the efficacy of the anti- EGFR antibody: T-CORE1201"
In addition, the running title has been revised as follows.
"Biweekly cetuximab and DNA methylation for colorectal cancer"

Reviewer B:
First, the title needs to indicate the two objectives of this study, the efficacy and safety of biweekly cetuximab plus mFOLFOX6 or mFOLFIRI as a second-line treatment and the prognostic role of DNA methylation status, and the clinical research design of this study, i.e., a prospective cohort study.

Reply: We changed the title to the following as per Reviewer A and B's suggestion.
"Phase II study of biweekly cetuximab plus mFOLFOX6 or mFOLFIRI as second-line treatment for metastatic colorectal cancer and exploratory analysis of associations between DNA methylation status and the efficacy of the anti- EGFR antibody: T-CORE1201"
In addition, the running title has been revised as follows.
"Biweekly cetuximab and DNA methylation for colorectal cancer"

Second, the abstract needs some revisions. The purpose did not indicate the knowledge gaps on the two research focuses and the clinical significance of this study. The methods needs to describe the inclusion of subjects, the assessment of baseline clinical factors and DNA methylation status, follow up procedures, and measurements of efficacy and safety outcomes. The results need to summarize the clinical

characteristics of the study sample. Please further report the HR and P values for the independent prognostic role of DNA methylation status. Because this is not a RCT and it remains unclear whether the prognostic role of DNA methylation status is independent, the authors need to tone down the current conclusion.

Reply: Thank you for your kind suggestions. We have modified the abstract as advised (see Page 3–4, line 47–85). We have added knowledge gaps on anti-EGFR treatment as second-line chemotherapy and DNA methylation status in the purpose, inclusion subjects, follow up procedures and measurements of efficacy and safety outcomes in the methods, and results of multivariate analysis in the Results section. Unfortunately, due to the word count limit, we couldn't add the assessment of baseline clinical factors in the methods, and summary of the clinical characteristics of the study sample in the Results section. Additionally, we have revised the statement in the conclusion in accordance with your and reviewer A's comments.

Third, the introduction of the main text needs to have a review on the knowledge gaps on the efficacy and safety of biweekly cetuximab plus mFOLFOX6 or mFOLFIRI as a second-line treatment and the prognostic role of DNA methylation status, as well the potential clinical contributions of the two research focuses.

Reply: We agree with your suggestion. We have added data to cover the knowledge gaps of biweekly cetuximab with chemotherapy (see Page 5, Line 126–140) and the DNA methylation status (see Page 6, Line 146–148) in the introduction. We have also modified our description of the Discussion to reflect the modifications made to the Introduction (see Page 15, Line 355).

Fourth, in the methodology of the main text, please describe the clinical research design, sample size estimation, assessment of baseline clinical factors, and follow up procedures. The authors need to explain why the current sample size is lower than the target number 100. The sample size estimation should also consider the analysis on the prognostic role of DNA methylation status. In statistics, please ensure $P<0.05$ is two-sided and use multiple Cox regression analysis to ascertain the independent prognostic role of DNA methylation status, because the clinical covariates would bias the DNA methylation-prognosis association.

Reply: Clinical research design was described in the research design in the Patients and Methods (see Page 7, Line 158–165). Sample size estimation and assessment of baseline clinical factors were described in the statistical analysis in the Patients and Methods (see Page 9, Line 208–216 and Line 217–218, respectively). We added a reason for why the study was closed without reaching the target number of participants (see Page 10, Line 228–229). In this study, the predictability of DNA methylation status on the efficacy of the anti- EGFR antibody-containing treatment was not a primary endpoint, but exploratory analysis. Therefore, it was not taken into account in the sample size estimation.
Further, we have added univariate and multivariate results for PFS and OS (Table 4) in accordance with your and Reviewer A's comments. Please refer to our response to Reviewers A's comments for details.