Author's response to reviews

Title: Modified FOLFOX-6 chemotherapy in advanced gastric cancer: Results of phase II study and comprehensive analysis of polymorphisms as a predictive and prognostic marker

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Editor

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Dear editor

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We wish to express our gratitude to you and the reviewers for the consideration and thorough review of our manuscript entitled “Modified FOLFOX-6 chemotherapy in advanced gastric cancer: Results of phase II study and comprehensive analysis of polymorphisms as a predictive and prognostic marker”. We have tried to revise our manuscript based on the reviewers’ comments as much as possible, which we feel has clarified and strengthened the paper considerably.

Here we have addressed the concerns of the reviewers on separate pages, as well as our responses to specific comments. I hope that you and reviewers will find these alterations satisfactory. We look forward to having our manuscript published in “BMC Cancer”.

Best wishes,

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Thank you for your interest and your valuable feedback. We revised the manuscript and yellow highlighted lines were marked in revised text. First of all, I would like to explain our purpose of this study.

In the past, the chemotherapeutic agents for gastric cancer were limited to several agents including 5-fluorouracil (5-FU), platinum and anthracycline. However, recently numerous agents including taxane, irinotecan and several targeted agents were also incorporated for gastric cancer treatment as 1st line or 2nd line chemotherapy. It remains to be clarified which is the best combination, with the highest efficacy and lowest toxicity. Although 5-FU remains the main chemotherapeutic agent, some patients do not respond to 5-FU based chemotherapy and we can’t predict this chemosensitivity yet. We also agree with the reviewers that the number of patient are relatively small to have an appropriate statistical power for lot of pharmacogenomic parameters including TS, ERCC, XPD and so on because the number of patients was calculated based on the primary endpoint of this study which is a response rate and 73 patients appropriate for this primary endpoint. The secondary purpose of this study was to find out the appropriate candidate of pharmacogenomic parameters associated with the prognosis of the patients who treated with 5-FU based therapy in homogenous population (1st line chemotherapy for relapsed or metastatic gastric cancer) treated with same regimen because we know some patients do not respond at all. In that case, we think we should try other regimen as 1st line chemotherapy. We can not use other ethnic data because we found several ethnic differences are existed and we have to find a candidate pharmacogenomic factor first to determine the appropriate target number for future prospective trial. The results of this study give us information about the candidate pharmacogenomic parameter for future trial. Based on this exploratory data, we designed a prospective study focusing on the value of TS 6-bp deletion as an independent factor in larger scale study which will include 404 gastric cancer patients. Please understand our
purpose of study, the primary endpoint was response rate of modified FOLFOX chemotherapy which does not use 5-FU bolus and the secondary endpoint was to find a candidate pharmacogenomic parameter for future trial which explain the exploratory nature of this study.

In the presented study, we suggested the prognostic value of TS 6-bp deletion polymorphism in advanced gastric cancer. However, the reviewers indicated the borderline significance of p-value. To support the biologic effects of the -6bp/-6bp in TS-3’UTR, *in vitro* experiment using gastric cancer cell lines was conducted. In our experimental results, gastric cancer cell line that had a -6bp/-6bp homozygotes tended to show lower TS protein expression and higher sensitivity to 5-FU. This *in vitro* data might explain why -6bp/-6bp polymorphism showed better clinical outcome to modified FOLFOX-6 chemotherapy. And there are several published data supporting that the -6bp/-6bp deletion polymorphism in the 3UTR of TS is associated with decreased mRNA stability *in vitro* and lower intratumoral TS expression in vivo and we described this in main text.

The followings are the comments of the reviewers and our responses to specific comments. Thank you very much for your consideration.

**Reviewer 1 : Dr. Salah-Eddin Al-Batran**

1. During the last 6 years, FOLFOX 6 FOLFOX 4 and several of their modifications were used for gastric cancer and were also published. Thus the results presented in this study do not add significantly to the knowledge already existing. The Pharmacogenetic results may be of significant value, but the study is, as I understood from the paper, primarily clinical. The study rational and the study methodology contain some limitations, e.g. the statistical hypothesis is very difficult to understand and must be changed.

Response>

To clarify the objective of this study, we revised the last part of the background to facilitate our study aim according to reviewer’s recommendation (in page 4, 3rd paragraph).

“*The primary endpoint of this study was to evaluate the efficacy in terms of response rate and the secondary endpoints of this study were the efficacy in terms of time to progression, overall survival and toxicity of modified FOLFOX-6 chemotherapy in AGC patients. Pharmacogenomic collateral study was performed to identify the predictive or prognostic value of germline polymorphisms of candidate genes associated with 5-FU and oxaliplatin.*”
The statistical hypothesis changed in order to clarify in page 6 line 11.
We also agree with the reviewers that the number of patient are relatively small to have an appropriate statistical power for lot of pharmacogenomic parameters including TS, ERCC, XPD and so on because the number of patients was calculated based on the primary endpoint of this study which is a response rate and 73 patients appropriate for this primary endpoint. The secondary purpose of this study was to find out the appropriate candidate of pharmacogenomic parameters associated with the prognosis of the patients who treated with 5-FU based therapy in homogenous population (1st line chemotherapy for relapsed or metastatic gastric cancer) treated with same regimen because we know some patients do not respond at all. In that case, we think we should try other regimen as 1st line chemotherapy. We can not use other ethnic data because we found several ethnic differences are existed and we have to find a candidate pharmacogenomic factor first to determine the appropriate target number for future prospective trial. We revised Table 5 and 7 in order to clarify at a glance. The results of this study give us information about the candidate pharmacogenomic parameter for future trial.

2. There are also many incoherencies that deserve the attention of the authors in the results, e.g. the rate of G3 neuropathy was 1.4%. This is much lower than the rates observed in other studies using Oxaliplatin at 100mg/m$^2$; e.g. Louvet et al. reported 21% G3 neuropathy with a similar schedule. Also the rates of nausea, vomiting, hepatic enzymes and others are not in line with results published previously.

Response>

In terms of toxicities, original FOLFOX-6 caused significant toxicities, including myelosuppression and peripheral neuropathy (Louvet C et al J Clin Oncol. 2002;20: 4543) as reviewer’s comments. In that study, the median cumulative doses were 901 mg/m$^2$ for oxaliplatin, which is much higher than our study (the median cumulative doses were 570 mg/m$^2$). In addition, eight of nine (89%) who experienced grade 3 neurotoxicity had a cumulative oxaliplatin dose of more than 1,000 mg/m$^2$ in original FOLFOX-6 regimen. Furthermore, in our study, if the patient experienced G2 neuropathy, oxaliplatin dose reduction to 85mg/m$^2$ was performed and this dose modification also contribute to the lower incidence of grade 3 neurosensory toxicity. Compared to original FOLFOX-6, both weekly and biweekly reduced-dose combinations of oxaliplatin/5-FU/ folinic acid without 5-FU bolus showed a more favorable toxicity profile with lower rates of myelosuppression and hepatotoxicity as shown in table.
### Table - FOLFOX in Advanced Gastric Cancer

<table>
<thead>
<tr>
<th>Author/Regimen</th>
<th>Regimen Details</th>
<th>Neutropenia</th>
<th>Hepatotoxicity</th>
<th>Neuropathy</th>
<th>Cumulative Dose of Oxaliplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With 5FU bolus</strong></td>
<td></td>
<td></td>
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<tr>
<td>Louvet C et al (J Clin Oncol. 2002;20:4543)</td>
<td>Oxaliplatin 100 mg/m² + FA 400 mg/m² + bolus 5-FU 400 mg/m² + 5-FU 3,000 mg/m² 46-hr</td>
<td>38%</td>
<td>NA</td>
<td>21%</td>
<td>901 mg/m² (Median 10 cycles)</td>
</tr>
<tr>
<td>De Vita F et al (Br J cancer 2005;92:1644)</td>
<td>Oxaliplatin 85 mg/m² + FA 200 mg/m² + bolus 5-FU 400 mg/m² + 5-FU 600 mg/m² 22-hr</td>
<td>36%</td>
<td>0%</td>
<td>5%</td>
<td>* Median 7 cycles</td>
</tr>
<tr>
<td><strong>Without 5FU bolus</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Lordick F et al (Br J Cancer. 2005;93:190)</td>
<td>Oxaliplatin 50 mg/m² + FA 500 mg/m² + 5-FU 2,000 mg/m² 24-hr</td>
<td>8%</td>
<td>0%</td>
<td>0%</td>
<td>800 mg/m² (Median 4 cycles = 16 weeks)</td>
</tr>
<tr>
<td>Al-Batran SE et al (J Clin Oncol. 2004;22:658)</td>
<td>Oxaliplatin 85 mg/m² + FA 500 mg/m² + 5-FU 2,600 mg/m² 24-hr</td>
<td>4.9%</td>
<td>2.4%</td>
<td>0%</td>
<td>595 mg/m² (Median 7 cycles)</td>
</tr>
<tr>
<td><strong>Our regimen</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin 100mg/m² + FA 100 mg/m² + 5-FU 2,400 mg/m² 46-hr</td>
<td>11.0%</td>
<td>0%</td>
<td>1.4%</td>
<td>570 mg/m² (Median 6 cycles)</td>
<td></td>
</tr>
</tbody>
</table>

*Gr 3 neurotoxicity developed at a dose of oxaliplatin ranging from 935 to 1275 mg/m²*

In our modified FOLFOX-6 regimen, we have omitted 5-FU bolus and prolonged 5-FU infusion hour (46hr) with low dose folinic acid. We obtained more tolerable toxicity profiles and we believed that this modification might contribute to tolerable toxicity. We think that less toxicity with same efficacy was the strong points of our regimen. We also have a clinical trial using same regimen for Korean FDA registration and it was presented in ASCO 2002 (Bang et al. Proc Am Soc Clin Oncol 2002;21:abstr # 2249). In that trial the G3/4 hepatotoxicity was 1/161 cycles (0.6%) and no G3/4 neurosensory toxicity was observed.

Figer A et al. reported the data compared FOLFOX 4 (oxaliplatin 85mg/m2 and 5-FU bolus 400 mg/m2) and FOLFOX 7 (oxalipaltin 130 mg/m2 without 5-FU bolus) in elderly (76 – 80 year old) CRC patient. Grade 3 neuropathy was more frequently observed in FOLFOX 4 than
FOLFOX 7 in patients less than 75 years old (18.2% vs. 13%). These data also showed the dose of oxaliplatin for 1 cycle is less important than cumulative dose and 5-FU bolus might have a role for sensory neuropathy.

Above mentioned data support that our G3/4 toxicity profiles were comparable to other modified FOLFOX regimen using lower dose of oxaliplatin or omitting 5-FU bolus infusion.

We described this at Discussion part in page 11 line 5.

“Grade 3 or 4 peripheral sensory neuropathy occurred in only 1.4% of the patients. This was lower than original FOLFOX-6 using oxaliplatin of 100 mg/m² with a median cumulative dose of 901 mg/m² for oxaliplatin. In our study the median cumulative dose of 570 mg/m² for oxaliplatin which is lower than original FOLFOX-6. With considering median cumulative dose of oxaliplatin, this was comparable to other lower dose oxaliplatin-based regimen or omitting the 5-FU bolus [4, 7]. In our study, dose modification of oxaliplatin to 85 mg/m² was performed if the patient experienced grade 2 peripheral neuropathy and we strictly followed the protocol which permitted the initiation of chemotherapy after recovery from all toxicities less than grade 2.”

Reviewer 2: Dr. Annamaria Ruzzo

General
The authors investigated the efficacy and toxicity of modified FOLFOX-6 chemotherapy in AGC patients. They also analyzed the predictive or prognostic value of polymorphisms of genes associated with 5-FU and oxaliplatin. The paper is interesting with positive results.

Major Compulsory Revisions
1. The authors do not mention how many cases are intestinal and diffuse gastric cancer. The two histological subtypes could influence the results.

Response>
  Some investigators observed differential patterns in the intestinal and diffuse types of gastric cancer. It would affect the results as reviewer’s comments. We had reviewed tumor specimens, and determined the histotype. The results were belows.
1) Diffuse 23 (31.5%)
2) Intestinal 47 (64.4%)
3) Unknown 3 (4.1%)
We described this in table 2. Genotype, response rate (RR), time to progression (TTP) and overall survival (OS) were not difficult according to histotypes (RR: $p=0.557$, TTP: $p=0.495$, OS: $p=0.392$, described in Table 5,6).

2. It has been used in the analysis the Bonferroni’s correction? If not, Why?

Response>
That is an important point because the correction for multiple comparisons in genome screening is important. A major controversy exists in determining significance levels for candidate gene or genome-wide association scans using single-nucleotide polymorphism (SNP) data. The number of individual tests can become very large and can lead to an inflated type I error rate. If the $p$-value is set to 0.05, there is a 5% error margin for each single gene to pass the tested. If 10,000 genes are tested, 500 genes might be found to be significant by chance. Hence Bonferroni correction is used in order to avoid false positive especially in genome screening.

In this study, we did not use Bonferroni correction. The reasons were belows:

1) The goals of our study are to evaluate the efficacy of modified FOLFOX-6 which omitting 5-FU bolus to minimize the myelosuppression in advanced gastric cancer population who has very limited survival duration and toxicity profile is very important for quality of short life span. And second goal was subsequently to identify the prognostic value of SNPs of genes associated with 5-FU and oxaliplatin in homougeous population for future trial to find out the population who should be treated with other agent which do not containing 5-FU in first line setting.

Our study is not large number genome wide screening which frequently requires multiple testing corrections such as Bonferroni. Genome screening is not ultimate goal of this presented study.

Hence viewpoint of the presented study would be understood from a different stand point. This study is exploratory rather than confirmative with $p$-value of 0.05. We mentioned this in discussion part, and this results require further independent prospective confirmation. We already designed the prospective trial using the result of this exploratory study in 404 gastric cancer patients.

2) Bonferroni correction is too strict and over-conservative. Because it is a single step method,
that is all the $p$-values from the various statistics are compared to the same benchmark value. Bonferroni correction has a limitation as we further increase the number of markers, as the assumption of independence between the markers becomes increasingly unrealistic and leads to an unnecessary loss of power. As we increased number of tested SNPs, corrected $p$-value becomes markedly smaller and the statistical power decreased rapidly. That means true positive SNPs are easily considered as false negative.

3) The investigated genes and SNPs in the presented study were identified in other studied conducted in colorectal cancer with biological supports. Investigated SNPs were not in terms of screening. However, we know ethnic difference was exist and we need our exploratory data in homogenous population for future confirmatory trial. Strict correction for multiple comparisons might be not needed in exploratory setting. Hence we thought that Bonferroni correction is not appropriate in our study at this time.

Minor Essential Revisions
1- Nomenclature should be corrected through the paper: example XPD Arg156Arg instead of C156A

Response>
We revised our manuscript as reviewer’s recommendation.

Reviewer 3: Dr. Manuel Perucho

General
I read the paper and I think it deserves further consideration. The opinion by the first referee was too negative and I disagree that the paper is scientifically unsound. The work nevertheless is borderline, as the association of the 6bp deletion at the Thymidylate Synthase (TS) gene with cancer progression requires further confirmation after reanalyzing the data by correcting with Bonferroni, as suggested by reviewer 2.

Response>
As we discuss under the reviewer 2’s response, we did not apply Bonferroni corrections in our study because we test 10 SNPs in this study and this is an exploratory study for searching candidate pharmacogenomic parameter for future large scale study. Instead of Bonferroni
corrections, we did multivariate analysis using Cox regression model including clinical factor and pharmacogenomic factor as a prognostic factor. Based on our result, we also revise the conclusion from “The 6-bp deletion in TS-3’UTR can be used to select patients who are likely to benefit from 5-FU based modified FOLFOX-6 in AGC.” to “The 6-bp deletion in TS-3’UTR might be a candidate to select patients who are likely to benefit from 5-FU based modified FOLFOX-6 in future large scale trial.”

We add the mention about the preclinical data reported by Mandola MV et al. (ref. 18) which is that -6 bp deletion polymorphism in the 3’UTR of TS is associated with decreased mRNA stability in vitro and lower intratumoral TS expression in vivo. Further, the 6 bp polymorphism varies greatly within different ethnic populations and is in linkage disequilibrium with the TS 5’ tandem repeat enhancer polymorphism.

In addition, to determine the effects of the -6bp/-6bp in TS-3’UTR to TS protein expression, in vitro experiment using gastric cancer cell lines was conducted. We investigate the genotypes of Korean gastric cancer cell lines, and we tried to find out their correlation with TS protein expression and 5-FU sensitivity. In our experimental result, gastric cancer cell line that had a -6bp/-6bp homozygotes tended to show lower TS protein expression (TS/tubulin expression ratio 0.88 in -6bp/-6bp vs.1.15 in +6bp/+6bp or +6bp/-6bp, p=0.095 by Mann-Whitney U test). Subsequent Western blot analysis showed lower TS expression was associated with higher sensitivity to 5-FU in our in vitro data (figure 3 in Kim JH, Im SA, Bang YJ et al. Cytotoxic effects of pemetrexed in gastric cancer cells. Cancer Sci. 2005 Jun;96(6):365-71, reference 34).

This biologic in vitro data might explain why -6bp/-6bp polymorphism showed better clinical outcome to modified FOLFOX-6 chemotherapy.

We described these in material and methods section (page 7, 2nd and 3rd paragraphs), results section (page 10, 2nd paragraph), discussion section (page 12, line2)
Figure. Linear correlation between TS protein expression and IC50 of 5-FU (p<0.002, \( r^2=0.887 \))

In addition-
1. We revised Table 5 and 7 in order to clarify at a glance.
2. We added two more references to explain statistics and experimental methods (reference number 34,35)
3. If you feel that there are too many table and figure, we are able to omit table and figure.