Author's response to reviews

Title: Genomic profile predicts the efficacy of neoadjuvant chemotherapy for cervical cancer patients

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Author's response to reviews: see over
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Professor Dafne Solera,
Executive Editor
BMC Cancer

Dear Professor Dafne Solera,

Thank you for your letter and for the reviewer’s comments on our manuscript. We are pleased with your interest in our paper, and are delighted that the referees evaluated our work favorably. We found the reviewer’s comments to be constructive, and we have revised the manuscript in close accord with their recommendations.

Our point-by-point responses to the comments are described below and the changes in the revised manuscript are colored in red for the convenience of the reviewers. As following reviewer’s comments, several analysis were conducted not only to support primary manuscript but to bring further findings. Consequently, several figures and supplementary materials were uploaded with descriptions in the main document.

We believe that the revised manuscript is substantially improved and hope that you find it suitable for publication in BMC Cancer.

Sincerely yours,

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Reviewer 1

1. **Sampling and intervention**
   
   The clinicopathological information of 38 LACC patients treated with NAC-RH\(^+\), however, Peripheral blood samples from 23 patients were used for genotype studying. Do the authors choose the 23 blood samples for some reason?

   We appreciate the careful review. We never intentionally selected patients. NAC-RH treatment has been operated for 38 LACC patients under the current protocol since 2007, but \(UGT1A1\) genotyping was introduced to our institute in 2010, and so recent 23 patients underwent \(UGT1A1\) genotyping in clinical setting.

2. **Microarray analysis**
   
   Can you comment why the samples are filtered by meeting the standard? Probes showing expression value >5.0 in at least one sample?.

   We have evaluated expression microarray data using this rather strict standard (Abiko K, et al. Clin Cancer Res. 2013; 19: 1363-74) as we think that gene expression analysis should be done with differentially expressed genes to draw reliable findings. Probes showing expression value < 5.0 were, therefore, excluded, and the item “For performing gene expression analysis with differentially expressed genes” was added in the Method part (page 8).

3. **Gene expression microarrays of 12 post-NAC tumors were analyzed.** Although the shrinking rate LVSI and \(UGT1A1\) polymorphisms are given in figure1, it would be better to comment more details about the characteristics of these 12 patients in supplementary data.

   It would be better to comment the abbreviation “NA” in figure 1, do you mean that the data of \(UGT1A1\) polymorphisms is lost in the sample? The NAC-responders and non-responders samples should be marked in figure1.

   In accordance with these helpful suggestions, we added the detailed clinical information of 12 cases in Supplementary Table 1 and remarks on Figure 1. Description for NA was also added to indicate patients who did not take \(UGT1A1\) genotyping test.

4. **GSEA analysis** revealed that several metabolism-related pathways were significantly up-regulated. Instead of others pathways, glutathione metabolism pathway (GMP) was chose? can authors explain the reason or their comments?

   Real time PCR should be performed to confirm the genes expression of Glutathione metabolism pathway in cancerous tissues.

   ssGSEA also showed that GMP scores of HCT-116 cells were significantly higher in CPT-11-resistant derivatives?, can you give the GMP scores performed in SCC cell lines by using the ssGSEA analysis?

   We thank the reviewer for these meaningful concerns. The prominent expression of GMP genes, \(GPX2\), \(GSS\), and \(GCLM\), was confirmed in NAC-responders by qPCR (Figure 2B). In our analysis, GMP was not only the most biologically significant pathway among metabolism-related pathways, but it was reported to be most highly associated with sensitivity of CPT-11 and CDDP. Among SCC cell lines, ssGSEA scores for other metabolism-related pathways were not correlated to IC50 values for these drugs (data not shown). As GMP scores of HCT-116 cells were significantly higher in CPT-11-resistant derivatives (Supplementary Figure 2B), GMP scores were relatively low in SCC cell lines sensitive to CPT-11 in a dose dependent manner (Figure 3A). These findings were added as figures and descriptions in the Results part (page 12-13).

5. **Since the authors found that there was a positive correlation between GSH and GMP in 4 cultured cervical cancer cell lines,** they should give detail information of the human cervical cancer cell lines (Ca-ski, SKGIIIA, Hela, and ME-180), do these cell lines resist toward CPT-11? When exposing to CPT-11, do the survival rate of the cervical cancer cells have a positive correlation with GSH and GMP scores? The authors
should perform experiments for supporting the hypothesis that there was a positive correlation between GSH and GMP in 4 cultured cervical cancer cell lines.

In response to the reviewer's comments, detailed descriptions for cervical cancer cell lines were added in the Methods part (page 8) as follows: All of them are representative cervical cancer cell lines and their gene expression microarray data could be obtained with IC50 values for CPT-11 from COSMIC dataset. Under the treatment of CPT-11, apoptosis was induced in Ca-ski cells and ME-180 cells with low GMP and/or GSH scores in a dose dependent manner. Along with high GSH concentration, GPX2 and GCLM were more highly expressed in Hela cells and SKGIIIa cells. These results were added in Figure 3A and Supplementary Figure 2C, respectively.

6. Discussion

Intriguingly, UGT1A1 polymorphism analysis revealed that there was UGT1A1*28 heterozygotic polymorphism in SKGIIIa cells (Figure 3B), which indicated a contribution of host UGT1A1 polymorphisms to CPT-11 susceptibility in tumors, however, the conclusion show that the UGT1A1 genotyping is detected in blood samples, this is somewhat misleading, can the authors explain the difference of UGT1A1 polymorphism between tumors, cell lines and blood samples.

We appreciate the careful review. It hasn't been reported so far that UGT1A1 polymorphism contributes to tumor phenotypes, but our cell line data indicated the potent contribution of host UGT1A1 polymorphisms to CPT-11 susceptibility in tumors as the reviewer pointed out. Although we assume that UGT1A1 polymorphisms of blood sample may coincide with those of paired tumor sample, UGT1A1 polymorphisms were only examined in blood samples of patients, and the difference of UGT1A1 genotype couldn't be compared between host and tumor. We thus added the comments in the Discussion part (page 17) as follows, to avoid misleading: We presumed that the UGT1A1 genotypes of cancerous tissues matched with those of host although there has not been any report thus far about the correlation of UGT1A1 polymorphism between tumor and host.

Reviewer 2

1. Just using the post-chemotherapy cancerous tissues to identify the predictive markers of chemotherapy efficacy was not available. Although the predictive value of screening markers (GMP score) was confirmed in cell lines, the authors also should test the value of GMP score in pre-chemotherapy biopsied samples. Or directly using the pre-chemotherapy biopsied samples to perform the genomic analysis stratified by the efficacy of CPT-11 plus Nadaplatin.

We appreciate this precise comment pointing out the limitation of our findings. We definitely agree that biopsied samples prior to chemotherapy would be more reliable to detect the biomarker, and have described this limitation in the Discussion part. It is a pity but we unfortunately had RNA extracted pre-chemotherapy biopsied samples, and there is no published microarray dataset of cervical cancer treated with this regimen. Nevertheless, unsupervised hierarchical cluster analysis of a web-published SCC gene expression microarray, GSE6213 which contains paired samples of identical patient before/after chemotherapy, revealed the following: Gene expression pattern was not remarkably affected by chemotherapy, since cluster dendrogram did not divide samples of each individual patient before/after chemotherapy (Supplementary Figure 3). We also performed in vitro assays with SCC cell lines for validating GMP activity (Figure 3A and Supplementary Figure 2C). These findings to support our data with post-chemotherapy samples were added in figures and descriptions in the Discussion part (page 16).

2. All 38 patients undergoing NAC-RH treatment, why only 12 patients had the cancerous tissue samples after surgery?
It is not due to our intentional selection but because of stocked sample quality. To avoid misleading analysis, the quality of retrieved RNA from each frozen tumor tissue sample was assessed by bio-analyzer, and only 12 samples satisfied the RNA integrity number more than 7.0. Thus, RNA analysis was performed with these 12 patients. This was added in the Method part (page 6).

3. “Prospective” or “retrospective” profile of this study should be stated. If the present study is a retrospective one, only 38 patients with LACC in about 5 years seemed to be insufficient. It is hard to believe this is a consecutive data.

In response to reviewers’ comment, we added the detailed description in the Method part (page 6). Total 209 cervical cancer patients underwent primary therapy for these 5 years. NAC-RH was applied on the patients who bore bulky tumors at stage Ib2 larger than 4cm or tumors at stage IIb without wish for radiotherapy, and 38 out of 209 patients met this criterion to undergo NAC-RH.

4. The evaluation method should be specified, “RECIST” or “WHO”. Moreover, why the authors defined “shrinkage less than 50%” as “resistance to CPT-11”? Why not “PD”? Furthermore, the criteria of RH after NAC chemotherapy should be specified.

We evaluated the size of tumor based on RECIST criteria, and the word “RECIST” was added in the Method part (page 7). We agree with the reviewer’s comment about the definition of resistance. The disease free survival was longer in patients with shrinkage > 50% than patients with shrinkage < 50% (p< 0.0021), while 30% shrinkage rate which defines PD did not discriminate the prognostic outcome in our cohort (p= 0.512). The reason why we set 50% as cut-off-value was added in the Result part (page 11). The criteria of RH after NAC was determined as described above.

5. What is the distribution of GMP score in 23 patients who were performed UGT1A1 genotyping analysis? A combined analysis of GMP score and UGT1A1 polymorphisms should be performed. Moreover, the statistical difference of response rates and side effects of NAC between UGT1A1 polymorphisms and wild type is not pretty convincing just in total 23 patients (11 cases vs. 12 cases) especially in a retrospective data. An enlarged sample size will be more helpful.

We also appreciate the concerns about UGT1A1 polymorphisms. In this study, RNA analysis was performed for 7 out of 23 patients whose samples showed satisfactory RNA quality. Among these 7 samples, 5 had UGT1A1 polymorphisms, and GMP score in this group was a little lower (1423.6) than the wild-type group (2497.2). This information was added in Supplementary Table 1. As described in the Discussion part, there were several reports on the correlation of UGT1A1 polymorphisms with adverse effects caused by CPT-11, but there was no report on the impact of UGT1A1 polymorphisms on the response rate in cervical cancer, which should be assessed in future prospective studies.

6. In colon cancer, UGT1A1*28/*28 polymorphism might mean more effective and also more toxicity than wild type. The present study indicated that UGT1A1 *1/*6 or *1/*28 polymorphism could predict good efficacy but less toxicity. The difference between these studies and the likely explanations should be discussed.

We also appreciate this constructive comment. It has been reported that patients with homozygous polymorphism had much higher AUC ratio of SN-38 than wild type patients, whereas patients with heterozygous polymorphism had slightly higher AUC ratio than wild type patients. This may be the reason why NAC containing CPT-11 showed higher efficacy without remarkable toxicity in the patients with heterozygous polymorphism. With gratitude to the reviewer’s suggestion, we added interpretation in the Discussion part (page 16-17).

Minor comments
Since all the patients underwent radical surgery, “DFS” should be used but not “PFS”. The p values below 0.05 were considered significant should specify one-side or two-side. The present study used microarray to analyze the gene expressions that should be “transcriptomics”.

We appreciate the careful review. We have revised the manuscript according to the reviewer’s comments.

“HCT-116 cell line” should be introduced in the “Methods” part.

We have explained in the Method part that Microarray data of HCT-116 cell line was obtained from Array Express: E-MEXP-1171.