Author's response to reviews

Title: Aberrant GSTP1 promoter methylation is associated with increased risk and clinical stages of breast cancer: a meta-analysis of 19 case-control studies

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Response to Reviewers' Comments

Dear Mr. Ryan Relox,

Thank you for processing our manuscript entitled “Aberrant GSTP1 promoter methylation is associated with increased risk and clinical stages of breast cancer: a meta-analysis of 19 case-control studies” (MS: 1043722986170152). We are delighted that it is potentially acceptable for publication in the BMC Cancer and we greatly appreciate the constructive and valuable comments from the reviewers. We have thoroughly revised our manuscript accordingly, and have highlighted all modifications in blue. Please find below our point-by-point responses to the reviewers’ comments.

Referee 1:

Comment 1. A first key issue is: only one of the studies (Brooks 2010, ref 25) did not find more aberrant methylation in cancer patients. Hence, what is the fundamental reason of performing the meta-analysis, as the result was expected, as essentially concordant in most studies?

Response: Thank you for your comments. As we know, "meta-analysis is the use of statistical methods to summarize the results of independent studies (Glass 1976)". According to the Cochrane Handbook of Systematic Review (http://handbook.cochrane.org/), the potential advantages of meta-analyses include an increase in power, an improvement in precision, the ability to answer questions not posed by individual studies, and the opportunity to settle controversies arising from conflicting claims. Therefore, the result of meta-analysis is more precise and has more reference than single study.

In our meta-analysis, as shown in the Figure 2, several studies showed that there were no statistically significant differences in methylation levels of GSTP1 between breast cancer patients and normal controls (including the study you
mentioned (Brooks 2010, ref 25)); however, other studies demonstrated statistically significant differences due existed. Owing to inconclusive and inconsistent results of these published clinical studies, we performed this meta-analysis to ascertain a more comprehensive and accurate association. Indeed, the pooled result of all included studies was significant (Figure 2).

Comment 2. An additional issue would, on the other hand, have required much more debate: why is blood DNA methylation expected to be a faithful indicator of promoter methylation in cancer?

Several studies have indicated heterogeneity in methylation patterns among different tissues for different genes.

If GSTP1 is being selected by the process of tumor progression, the more so if higher association is with late stage disease, it should be experimentally defined why methylation differences are expected in DNA from cells that had not been subjected to such a selection.

Not surprisingly, the negative study cited above was only conducted on blood cell DNA.

Consistent, the meta-analysis itself finds much lower hazard ratios in PBL than in cancer (OR=4.02, 95%CI=1.12-14.38), and CI border the value of 1.

Response: Thank you for your comments. According to your suggestion, we added the relevant text in the Discussion section (Page 8, line 202 to 216 and line 227 to 229).

As shown in Table 2, subgroup analyses, which were stratified according to the patients’ ethnicity, sample type and detection methods were performed to explore potential sources of heterogeneity and the differences among them. After stratified by sample type, we found that aberrant methylation of GSTP1 was correlated with the risk of breast cancer detected in tissue (OR=10.32, 95%CI=5.97-17.85) as well as blood samples (OR=4.02, 95%CI=1.12-14.38). Moreover, a high concordance between tumor and blood DNA methylation of GSTP1 was found in studies conducted on paired tumor tissue and blood samples from breast cancer patients (ref 27, 31). Yamamoto et al compared the gene methylation status in serum DNA before and after surgery in patients with primary breast cancer, and demonstrated that the origin of blood methylated DNA was the tumor tissue because patients with aberrant GSTP1 methylation in serum DNA collected before surgery were found to be negative for gene methylation after surgery (ref 31). This indicated that blood DNA methylation of GSTP1 could reflect alterations in the tumor and the ease of obtaining blood samples makes it a potential biomarker for diagnosis of breast cancer. In the present meta-analysis, the small number of patients, various ethnicity groups and different time of sample collection may contribute to relatively extended confidence intervals. (line 202-216)

Epigenetic alterations including DNA methylation and histone modifications which occur in transformed cells are identified as an early event during tumor development (ref 33, 37). Aberrantly methylated genes are frequently found in human cancers but rarely or not in normal controls, they are not limited to
patients with metastatic cancer but are also present in body fluid from patients with early or organ-confined tumors (ref 26). Our results showed that the methylation level of GSTP1 increased significantly in late-stage compared to the early-stage breast carcinomas, suggested that breast cancer patients with GSTP1 promoter hypermethylation may have a biologically aggressive phenotype. (line 227-229).

Comment 3. An additional reason of worry is the detection method. Best-quality results are expected from quantitative methylation analyses. These provided by far the smallest HR (Quantitative: OR=4.73, 95%CI=1.84-12.12). Highest associations were found for Semi-quantitative (OR=10.33, 95%CI=3.32-32.10) and Non-quantitative: OR=12.55, 95%CI=5.72-27.55) analyses, but seemed to have no impact on the conclusions of the study.

Response: Thank you for your comments, we have added the relevant text to explain the issue according to your comment (Page 8, line 219 to 226).

Since different methylation assays were applied to detect the methylation levels of GSTP1 in the studies included in this meta-analysis, we performed subgroup analysis based on methods to explore potential sources of heterogeneity and the differences among them. The results showed that aberrant methylation of GSTP1 was significantly correlated with the risk of breast cancer detected by quantitative, semi-quantitative and non-quantitative techniques, suggested these methods have the same effect in GSTP1 methylation detection.

Several papers have compared MS–MLPA (semi-quantitative) with pyrosequencing (quantitative) or MSP (non-quantitative) and showed a good concordance between MS–MLPA and pyrosequencing (ref 28, 35). However, different patient materials and sample size and the choice of different primer sets between different studies may influence the results. (line 219-226)

Comment 4. When only quantitative analyses of GSTP1 promoter methylation in blood DNA are pooled, is association of promoter methylation and cancer cases still confirmed?

Response: Thank you for your kindly suggestion. We performed this analysis according to your suggestion which based on 3 studies with 173 cases and 153 controls were finally analyzed and no significant association was observed (OR=2.92, 95%CI=0.32-27.11). However, the sample size is too small that may lead to false-negative results. Future studies that include a larger number of patients need to be conducted to clarify this important issue.

Comment 5. Publication bias was found by this analysis, but was not discussed, and had no impact on the conclusions of the study.

Response: Thank you for your comments. We have discussed this issue according to your suggestion (Page 9, line 254-257) as follows: "Only published clinical studies were selected in this meta-analysis, some unpublished and negative studies may contribute to publication bias. Since studies with statistically positive results were easier to publish than those with negative results, publication bias is inevitable."
Comment 6. HR for late-stage disease appear ‘reversed’. As increased association is with late stage disease a better presentation of these findings would be late stage/early stage ratios, for increased HR as associated with late stage.

Response: Thank you for your good comment. We have revised them accordingly in the manuscript (Page 2, line 41; page 6, line 161) and in Figure 3.

Comment 7. When removing the study by Brooks et al, 2010 heterogeneity measures decrease. This is almost tautologic (this was the only negative study included) and should be avoided.

Response: Thank you for your comment. Our results showed a moderate heterogeneity existed in investigating the correlation of GSTP1 methylation and breast cancer risk detected in blood samples and quantitative method by subgroup analysis (Table 2). Thus, sensitivity analysis was conducted by omitting a single study in each turn to see whether a particular omission could influence the overall estimate. After removal of the study by Brooks et al (ref 25), the heterogeneity decreased in some degree, however, the pooled ORs were not significantly changed, suggested the stability of our results.

Referee 2:
Comment 1. In the abstract will add more data,
Overall, the pooled results indicated that aberrant GSTP1 promoter methylation was significantly associated with the risk of breast cancer (OR=7.85, 95%CI=5.12-12.01, Caucasians OR=7.23, 95%CI=3.76-13.90 and Asians OR=11.71, 95%CI= 5.69-24.07).

Response: Thank you for your suggestion. We have added these data in the Results section of the Abstract.

Comment 2. In the result, the author can analyze more about the data of OR that include the 11 articles without control group for more number of case group to show that differ from present result or not.

Response: Thank you for your suggestions and we have revised the relevant text. To investigate the relationship of GSTP1gene promoter methylation with breast cancer onset risk, relevant case-control studies were selected in this meta-analysis. All included studies used normal samples as controls, which were composed of normal breast tissues from breast cancer patients and normal samples from non-cancer people. Among the 11 articles without control group, although some studies investigated the relationship between GSTP1promoter methylation and clinicopathological features of breast cancer patients, such as age, subtype and hormone receptor status, they do not analyze its relationship with tumor stage or histological grade. As these studies do not meet our inclusion criteria, thus were not included in the present analysis. (line 85)

Editorial requests:
1. Introduction header should be renamed as "background"
2. Please provide a titled conclusion
3. Please include a list of abbreviations section after the conclusion.
4. Please insert acknowledgments after the author contributions section.
Response: Thank you for your comments. We have revised accordingly in the manuscript.