Author's response to reviews

Title: A Case-Control Study of GST Polymorphisms and Arsenic Related Skin Lesions

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Author's response to reviews: see over
Reviewer's report
Title: A Case-Control Study of GST Polymorphisms and Arsenic Related Skin Lesions
Version: 1 Date: 27 June 2006
Reviewer: David J. Thomas
Reviewer's report:
General
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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1- The design of the study is described as a case/control comparison but it is not, as the authors note on page 6, a standard case/control design. The authors need to provide more detail on how the controls that are also exposed to arsenic in drinking water are differentiated from the cases that apparently have skin lesions. Also, are there controls with skin lesions? Stating that the design is valid and highly efficient is not sufficient to explain the design or to allay concerns of the reader. This issue must be addressed in the Methods.

Response: We agree that the study design is not a traditional case control study in that the main effects between arsenic concentration and skin lesions cannot be assessed; however formally the study design is still classified as a case control study. By definition, cases and controls are defined on the basis of outcome not exposure, cases have the outcome of interest while controls do not have the outcome of interest. This is better clarified in the text per Dr.Thomas’s suggestion. It is non traditional in the sense that the exposure distribution among controls were predetermined in order to ensure heterogeneity of exposure, and avoid overmatching on exposure vis a vis residence. The real purpose of this study was to look at the effect of potential modifiers on the exposure (arsenic) disease (skin lesions) relationship. In ensuring the distribution included enough low exposure subjects, the main association between arsenic concentration and risk of skin lesions would be biased as shown in the sensitivity analysis. The factors not directly related to well arsenic concentration are not impacted by this control selection, and the design allows us to study the impact of diet, genetic polymorphisms, urinary methylation capacity and other factors without bias.

Controls are defined as healthy individuals diagnosed as free arsenic related disease randomly selected in a 1:1 ratio from Pabna, age of at least 16 years, living in the same village as cases but not sharing a tube well. We did make a change on page 6 and specify that arsenic related diseases included skin lesions, per Dr Thomas’s suggestion.

In a previously published paper in EHP we showed that while estimates for the main effect of arsenic exposure on skin lesions is biased by control selection, we showed through sensitivity analysis that effect estimates for non- well arsenic concentration related variables remained stable even if we varied the percentage of controls selected with well arsenic concentration less than 50µg/l.
We will attach similar figures for the sensitivity analysis in this manuscript. The full description of the statistical methods for this analysis is in the manuscript referenced below and is available full text online. We have attached a copy of this manuscript with the comments, which provide the figures for the sensitivity analysis (McCarty et al, 2006).

We understand the point that the efficiency of the study is based on personal communication with the main statistician/co-author for the paper (Dr Louise Ryan, Professor and Chair of the Biostatistics Harvard School of Public Health Boston, MA USA). Dr Louise Ryan is a well known statistician and was a member of the 1999 Subcommittee on Arsenic in Drinking Water as part of the National Research Council (NRC, 1999). Dr Ryan has substantial expertise in this area. Dr Ryan has completed some theoretical work that shows that our design works efficiently for the purpose of studying effect modifiers. Since this theory has larger implication for other studies, we feel it is important for this work to stand alone as a methods paper, and we do not wish to include it as an appendix for this paper. The paper is currently written and will be in submission to an epidemiology journal.

Since we do agree with the reviewer that more statistical information is necessary, results of the sensitivity analysis that support the stability of the effect estimates for the main effect of the GST polymorphisms has been included. As one would suspect, effect estimates of variables not directly related to well arsenic concentration are stable and not biased by the control selection. In the case of this paper, status of GST genotype is stable as it would not be affected by well arsenic concentration. We can add a reference to this paper if the reviewer/editor wishes, but we believe the sensitivity analysis also demonstrates the stability of the effect estimates for GSTT1 and GSTP1.

This information and reference to the sensitivity analysis (Mc Carty et al, 2006) has been added to the text per Dr Thomas’s suggestion to provide more information on the statistical methods. We agree with your point that we needed to include more statistical support, and we have now included results of the sensitivity analysis to demonstrate the stability of the study design to look at modifiers of the exposure disease relationship.

References:


National Research Council (U.S.). Subcommittee on Arsenic in Drinking Water.

2- The analytical procedures used must be described in more detail. The paragraph on page 7 describing water collection and analysis is not intelligible. Also, if toenail arsenic concentrations were used in this study, then one must describe collection, processing, and analysis.

Response: At the bottom of page 7, we further defined “High” and “low “arsenic wells by their definition of < 50 µg As/l and ≥ 50 µg As/l for clarification. We did collect and analyze toenails for this study. Since we do not report the results in this manuscript we have removed all mention of the nails from the manuscript.

Further description was provided that previous BGS (British Geological Society) and World Health Organization (WHO) water arsenic concentration studies had previously measured a large percentage of wells in Bangladesh. A uniform system was developed to identify wells above and below the 50 µg/l drinking water standard in Bangladesh. Well found to be above the standard were previously marked with a red handle. Low exposure wells were marked with a green handle. A brief explanation of the handles was given in order to explain that while the interviewer knows generally knows the subjects is from a “high” or “low” area, the interviewer has no idea of the actual exposure concentration of the well until after the subject has been enrolled and the water has been analyzed by ICP-MS in the US.

3- For clarity and accuracy, the authors should avoid stating that the formation of arsenic-glutathione complexes is dependent on the catalytic activity of some glutathione transferase. As far as I am aware, there is no direct evidence that the reaction is catalyzed by this enzyme. Certainly expression of glutathione transferases affects the kinetic behavior of arsenic but this does not prove the action depends on catalysis of conjugation. Although this issue does not go to the validity of the data reported here, an unsubstantiated hypothesis for the mode of action of the glutathione transferases may mislead readers.

Response: We may have overstated the findings by Xie et al. (2004). We did not intend to mislead the reader and have made changes to acknowledge the point that the action of this process may not depend on the catalysis of the conjugate. We made a change to reflect the wording of Xie et al. It is now clearly stated that “GST activity catalyzes the formation of arsenic-GSH conjugates” and there is no statement within the text that action depends on the catalysis of the conjugate. Thank you for pointing out this clarification.

4- The description of the change in a commonly held opinion about the role of metabolism in the actions of arsenic is not a paradigm shift. Thomas Kuhn's careful description of a real paradigm shift (e.g., the Copernican revolution) describes a global change in thinking about a fundamental characteristic of the physical world. In the case of assessing the role of metabolism of arsenic, what occurred was self-correcting process in which investigators tried to falsify a hypothesis (that metabolism of arsenic is only detoxification) and found they could do so. Here, paradigm shift is scientific hyperbole.

Response: The term “paradigm shift” has been removed from the manuscript.

5- Table 2 contains an error in the footnote. I believe that both arsenic concentration in well water and arsenic concentration in nails were used for adjustment of the crude model.

Response: Nails were not adjusted for in the main model. In an earlier draft, we used two models- one adjusting only for well arsenic concentration and one adjusting for nail arsenic as a biomarker of exposure to well arsenic, but we have removed it from the manuscript without removing reference to it. Thank you for noting that sentence. All mention of nails has been removed from the manuscript since we did not discuss the results in this paper. Thank you for pointing out that nail arsenic concentration was noted in the table.

Reviewer's report
A Case-Control Study of GST Polymorphisms and Arsenic Related Skin Lesion
Title: s
Version: 1 Date: 10 July 2006
Reviewer: Alison Shield
Reviewer's report:
The authors have written an interesting paper reporting modest associations between several GST polymorphisms and arsenic related skin lesions. A number of studies have been undertaken in arsenic exposed populations at the association of various cancers with GST polymorphisms, including a recent similar paper investigating the GSTM1, GSTT1 and GSTP1 polymorphisms and skin lesions (Ghosh et al. Int J Cancer 118: 2470-2478, published online 13 Dec 2005). Main differences between the current paper and the published articles include the size and location of the study population. Generally the study design and analyses are acceptable, however a few comments still need to be addressed prior to publication and are listed below.

Response: Thank you. We appreciate Dr Shield’s comments and suggestions. We have incorporated the comments into the manuscript and into the responses below.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. The referencing in the introduction is poor as three sentences in the second paragraph are not referenced (beginning with “In vivo studies...” and ending “formation of arsenic-GSH conjugates...”). Also reference 13 is stated to include animal data that shows GSH-conjugates being transported by MRP “this reference does not show the stated data. The appropriate references should be included.

Response: We have corrected the reference in regards to 13 and used the primary sources. Reference 13 remains since it supports our, and others, findings. Additional references were added for the other two sentences in the paragraph.

2. It is not entirely clear to the reader why the authors have chosen to study GSTT1, GSTM1 and GSTP1 polymorphisms. Arsenic biotransformation remains a complex mechanism, however it is generally accepted (since 2001) that GST01 is the rate-limiting enzyme for the GSH mediated reduction of arsenic. There is no evidence that GSTT1 or GSTM1 are involved in the conjugation of GSH with arsenic (or its transport). Although the authors cite the possibility of increased GST activity being associated with saturation of MRP and hence accumulation of arsenic the paper cited shows only an increase in GSTP1 levels with no change in GSTT1. The authors need to provide a clearer rationale for their choice of polymorphic candidates and why other polymorphic GSTs (such as the omega class) have been excluded.

Response: At the time we executed the project the GSTT1, GSTM1 and GSTP1 polymorphisms were identified mainly due to their role in Phase II metabolism of exogenous and endogenous metabolism. Additionally, GST01 polymorphism analysis is more complex, as the variants are not simple SNP’s. Finally, the story as to GST01 role in As remains less than settled, as discussed below. GST01 was described and localized in 2003 (Whitbread et al., 2003). Recent genetic linkage studies have implicated polymorphism in the Omega class GSTs as a contributing factor influencing the age at onset of both Alzheimer’s and Parkinson’s diseases (Li et al., 2003; Kolsch et al.,2004). Polymorphisms that cause amino acid substitutions or
deletions in the Omega class GST have recently been investigated in a number of laboratories (Marnell et al., 2003; Whitbread et al., 2003). However, studies on cancer are sparse (only one very small sample size study from Thailand, Marahata et al, 2005).

The function of GSTO1 gene is unknown, and our research work is hypothesis driving and only focused on the SNPs with well described function changes.

There are several polymorphisms in GSTO1 and studies in the biologic function of GSTO1 are still sparse. A recent study failed to detect the 3 reported sequence variations in exon 6 for the GSTO1 gene at all in their study population, while only reporting a good distribution in prevalence in the polymorphisms at codon 4 (Ala140Asp). While they did detect the polymorphism at codon 4 (Glu155Glu), the detected distribution is almost entirely the Glu/Glu form and the polymorphism has a very low prevalence. (Kolsch et al, 2004).

We have clarified in the text that the reason for the choice of GSTT1, GSTM1 and GSTP1 was originally that polymorphisms in these genes have been associated with several cancers and we wished to see if it was associated with increased risk of skin lesions given arsenic exposure. Clarification has been made so that it is clear in the aims to main purpose was the investigate the effects of GSTT1, GSTM1 and GSTP1 polymorphisms in regards to risk of skin lesions given their previous associations to many cancer and other outcomes. Discussion of toxicological findings that support some association related to arsenic are included in the discussion section.

References:


Whitbread AK, Tetlow N, Eyre HJ, Sutherland GR, Board P. Characterization of the human Omega class glutathione transferase genes and associated polymorphisms. Pharmacogenetics. 2003 Mar;13(3):131-44.


3. In the methods section the authors have not clearly stated for the null polymorphisms how the genotypes were analyzed. Have you modeled on allele frequencies, null homozygotes vs. pooled heterozygotes and WT, or pooled null homozygotes and heterozygotes vs. WT? Why haven't alternative models been discussed â€“ we might expect to see a dose response for 2 vs. 1 vs. 0 copies of the WT allele. Similarly, it is not clear from Table 1 whether we are looking at allele frequencies or genotypes (and why is this different for GSTP1).

Response:

We have specified in the text that these are genotypes in Table 1 for GSTM1 and GSTT1 and allele frequencies for GSTP1, as suggested by Dr Shield.

We thank you for the comment and we have specified in the text that for the deletion polymorphisms GSTT1 and GSTM1, we have modeled them as homozygous wildtype and heterozygotes vs. homozygote null. We have clarified this in the text- thank you for the suggestion.

No alternative models were discussed for GSTT1 and GSTM1 because previous work had indicated the grouping in this manner. (Zhong et al, 1993). Previous papers have reported that the proper coding based on enzyme function is that heterozygotes and homozygote wildtypes product the same amount of enzyme and are grouped together vs wildtype ( Zhong et al, 1993). This method has been used previously in several studies from the group/our lab (Wang et al, 2004; Chen et al, 2004; Zhai etal, 2002)

There appears to be some discrepancy in the literature to coding based on the paper by Zhong et al is 2006 paper; however we grouped our GSTT1 and GSTM1 in the same manner as Ghosh et al. so that the results are directly comparable.
We chose to model \( GSTP1 \) in a way that allowed us to investigate whether there was a difference in enzyme expression between the heterozygote variant and the homozygote variant might be associated with risk of developing skin lesions. This decision was based on previously published papers that showed that polymorphisms in \( GSTP1 \) have shown different levels of enzyme expression for the heterozygote and homozygote variant genotypes. Watson et al showed that the \( GSTP1 \) polymorphism is a single base pair substitution where adenine is replaced by guanine resulting in an amino acid change in which isoleucine \( (I_{105}) \) is replaced by valine \( (V_{105}) \), possibly resulting in lower enzyme activity (Watson et al, 1998). CDNB conjugating activity measured for the \( Ile/Ile \) genotype group was significantly different from that observed in the \( Ile/Val \) group \( (P = 0.03) \), and from \( Ile/Val \) and \( Val/Val \) genotypes combined \( (P = 0.009) \) (Watson et al, 1998). Additionally Zhong et al has reported differences in enzyme expression associated with \( GSTP1 \) polymorphisms.

References:


4. Not being a statistician it is difficult for me to judge how robust the non-standard case-control design is – this should be assessed by an expert statistician. It would also be
useful to include a reference to a study using a similar approach rather than personal communications with one of the authors.

Response: We agree that we need to show more evidence of our rational. The sensitivity analysis that demonstrates the stability of our effect estimates taking into account the selection of controls (McCarty 2006).

From a biological standpoint, there would be no reason to believe the distribution of polymorphisms in GSTT1, GSTM1, or GSTP1 would be biased in any way by our selection of controls, and this is supported by the sensitivity analysis results. Water arsenic concentration is not related to frequency of GST polymorphisms. Selection of controls was done to ensure that we had a control population that represented the reported background exposure of the Pabna population (based on British Geological Survey results). We wanted to avoid matching our cases and controls on exposure (i.e., sharing a well), because this would also increase the chance that we matched on modifiers (family members may have similar genetics backgrounds, diet, SES, etc.). This study design allowed us to better investigate modifiers of the exposure disease relationship without matching on those factors inadvertently.

From a statistical standpoint, in a previously published paper in EHP we showed that while estimates for the main effect of arsenic exposure on skin lesions is biased by control selection, we showed through sensitivity analysis that effect estimates for non-well arsenic concentration related variables remained stable even if we varied the percentage of controls selected with well arsenic concentration less than 50µg/l. We will attach similar figures for the sensitivity analysis of GSTT1, GSTM1 and GSTP1 in this manuscript. The full description of the statistical methods for this analysis is in the manuscript referenced below and is available full text online.

We have attached a copy of this manuscript with the comments, which provide the figures for the sensitivity analysis (McCarty et al, 2006).

We do agree with Dr Shield’s suggestion that more information needed to be included regarding the statistical methods. The results of the sensitivity analysis which support the stability of the effect estimates for the main effect of the GST polymorphisms. As one would suspect, effect estimates of variables not directly related to well arsenic concentration are stable and not biased by the control selection. In the case of this paper, status of GST genotype is stable as it would not be affected by well arsenic concentration. We can add a reference to this paper if the reviewer/editor wishes, but we believe the sensitivity analysis also demonstrates the stability of the effect estimates for GSTT1, GSTM1 and GSTP1.

5. The sentence in paragraph 2 of the discussion is overstated; we found that there was an increased risk of skin lesions potentially greater MRP activity. No evidence has been found for associations between GSTT1 and MRP (see also comment 2). More compelling evidence for GSTT1 needs to be given in the discussion particularly given an OR of only 1.56 and a null result in the competing Ghosh paper. Many of these enzymes belong to families with overlapping substrate specificities and are therefore it is likely that the null polymorphism is compensated for.

**Response:** Thank you for the correction. The text has been corrected to state there is no evidence for GSTT1.

Ghosh et al may not have been able to observe a statistically significant effect for GSTT1 and GSTP1 for the reason of their small sample size and lack of power to identify this association. We have a much larger sample size and did not detect an association with GSTM1, while we did detect associations with GSTT1 and GSTP1.

As noted by the tables in Ghosh, 53.33% of their asymptomatic patients were GSTM1 negative, and 46.67% of their symptomatic patients with skin lesions were GSTM1 null. Controls were 42.51% GSTM1 wildtype, and Cases were 57.49% wildtype. We reported the frequencies of the SNPS to be similar for GSTM1 in that 41.1% of our controls were GSTM1 null and 41.0% of our cases; 58.9% of our controls were GSTM1 wildtype and 59% of our cases. Our prevalence of genotype SNPS was similar to Ghosh et al.

Ghosh et al coded GSTT1 in the same manner as GSTM1 and again those with the partial deletion of GSTT1 have been shown to produce no enzyme activity and the coding should have been (-/-) and (+/-) vs (+/+) which is the same way that they were coded in our study.

Additionally Ghosh et al had only 22 controls and 33 cases who were homozygote null (-/-), and 156 controls and 211 cases who were (+/-) or (+,+). They may not have had an adequate sample size to detect a significant association for GSTT1.

For GSTP1, they had even fewer subjects which would not have allowed the ability to detect an association. In this manuscript, we reported that individuals with the GG genotype (Val/Val) had a 1.86 increased odds of skin lesions compared to those individuals with the GSTPIAA genotype (OR 1.86 (95%CI 1.15-3.00). Ghosh et al reported having 3 cases and 3 controls with the (Val/Val) or GG genotype so they lacked the power to detect the association. From a statistical standpoint, having less than 5 observations in a cell should have resulted in an unstable model and this should not have been attempted.
We thank Dr Shield’s for bringing up this paper and we believe that including it in the discussion was very helpful.

6. It would be useful for the authors to compare their findings with those of Ghosh et al; who did not find differences in GSTT1 and GSTP1 genotypes but did find a significant difference for GSTM1.

Response: Thank you. We agree that this was important to include in the paper. We addressed these issues in response 5 and within the discussion of the text. We believe its discussion has added support to our findings, and also to stress the potential limitation of these SNPs being in Linkage disequilibrium with other important yet unidentified SNPs.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
1. “Along” has been superscripted for no apparent reason in the introduction.
Response: this did not come through in the file.
2. You refer in the text to arsenic toenail concentration in Table 2. It is not clear in your methods how this was determined (or how it is applied in Table 2 analysis).

Response:
Thank you. We have taken this suggestion into account and removed all reference to had measured toenail arsenic concentration. It was in the table to validate that current exposure was reflective of the biomarker of past exposure (toenails). Since we did not discuss the nails in this paper they have been removed from the manuscript.

3. I could not find where you have defined the abbreviation LRTs.

Response: Thank you. We have inserted “Likelihood Ratio Test” in the text. This has been corrected on page 12.

4. It is confusing to talk about GSTs in the context of methylation â€œ clarity about the role of GST in arsenic metabolism needs to be improved in the following discussion sentence: â€œâ€˜while only GSTO1-1 has been implicated in arsenic methylationâ€’â€˜TMâ€˜â€™
Response: We have clarified this in the revised text.

5. In the discussion â€œglutathione-Î½,transferaseâ€’Î½ needs to be corrected to glutathione transferase Î½,.
Response: Ok.

Discretionary Revisions (which the author can choose to ignore)
1. Interestingly the active ingredient in betel nut inhibits various GSTs â€“ it is possible that this may also be contributing to disease.

Response: This is a good point. We did report previously the main effects of reported that betel nut use was associated with risk of skin lesions (Mc Carty et al, 2006 EHP);
however we did not find any evidence in this population. We did assess the GST-Betel nut relationship and found no evidence of effect modification by betel nut use.

Unable to decide on acceptance or rejection until the authors have responded to the majoWhat next?: r compulsory revisio
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Reviewer's report
A Case-Control Study of GST Polymorphisms and Arsenic Related Skin Lesion
Title: s
Version: 1 Date: 30 June 2006
Reviewer: Ricardo Marcos
Reviewer's report:
General
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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
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X Discretionary Revisions (which the author can choose to ignore)
What next?: Accept after discretionary revisions
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.

Response: There are no specific changes suggested by Dr. Marcos. We thank him for this review and support.