Does Occupational Exposure to Solvents and Pesticides in Association with Glutathione S-Transferase A1, M1, P1, and T1 Polymorphisms Increase the Risk of Bladder Cancer? The Belgrade Case-Control Study

Marija G. Matic1,5*, Vesna M. Coric1,5*, Ana R. Savic-Radojevic1,5, Petar V. Bulat2,5, Marija S. Pljesa-Ercegovac1,5, Dejan P. Dragicevic3,5, Tatjana I. Djukic1,5, Tatjana P. Simic1,5, Tatjana D. Pekmezovic4,5*

1 Institute of Medical and Clinical Biochemistry, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, 2 Institute of Occupational Health, Belgrade, Serbia, 3 Clinic of Urology, Clinical Center of Serbia, Belgrade, Serbia, 4 Institute of Epidemiology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, 5 Faculty of Medicine, University of Belgrade, Belgrade, Serbia

Abstract

Objective: We investigated the role of the glutathione S-transferase A1, M1, P1 and T1 gene polymorphisms and potential effect modification by occupational exposure to different chemicals in Serbian bladder cancer male patients.

Patients and Methods: A hospital-based case-control study of bladder cancer in men comprised 143 histologically confirmed cases and 114 age-matched male controls. Deletion polymorphism of glutathione S-transferase M1 and T1 was identified by polymerase chain reaction method. Single nucleotide polymorphism of glutathione S-transferase A1 and P1 was identified by restriction fragment length polymorphism method. As a measure of effect size, odds ratio (OR) with corresponding 95% confidence interval (95%CI) was calculated.

Results: The glutathione S-transferase A1, T1 and P1 genotypes did not contribute independently toward the risk of bladder cancer, while the glutathione S-transferase M1-null genotype was overrepresented among cases (OR = 2.1, 95% CI = 1.1–4.2, p = 0.032). The most pronounced effect regarding occupational exposure to solvents and glutathione S-transferase genotype on bladder cancer risk was observed for the low activity glutathione S-transferase A1 genotype (OR = 9.2, 95% CI = 2.4–34.7, p = 0.001). The glutathione S-transferase M1-null genotype also enhanced the risk of bladder cancer among subjects exposed to solvents (OR = 6.5, 95% CI = 2.1–19.7, p = 0.001). The risk of bladder cancer development was 5.3-fold elevated among glutathione S-transferase T1-active patients exposed to solvents in comparison with glutathione S-transferase T1-active unexposed patients (95% CI = 1.9–15.1, p = 0.002). Moreover, men with glutathione S-transferase T1-active genotype exposed to pesticides exhibited 4.5 times higher risk in comparison with unexposed glutathione S-transferase T1-active subjects (95% CI = 0.9–22.5, p = 0.067).

Conclusion: Null or low-activity genotypes of the glutathione S-transferase A1, T1, and P1 did not contribute independently towards the risk of bladder cancer in males. However, in association with occupational exposure, low activity glutathione S-transferase A1 and glutathione S-transferase M1-null as well as glutathione S-transferase T1-active genotypes increase individual susceptibility to bladder cancer.

Citation: Matic MG, Coric VM, Savic-Radojevic AR, Bulat PV, Pljesa-Ercegovac MS, et al. (2014) Does Occupational Exposure to Solvents and Pesticides in Association with Glutathione S-Transferase A1, M1, P1, and T1 Polymorphisms Increase the Risk of Bladder Cancer? The Belgrade Case-Control Study. PLoS ONE 9(6): e99448. doi:10.1371/journal.pone.0099448

Editor: Keitaro Matsuo, Kyushu University Faculty of Medical Science, Japan

Received December 4, 2013; Accepted May 15, 2014; Published June 10, 2014

Copyright: © 2014 Matic et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Ministry of Education and Science of the Republic of Serbia (Grants number: 175052 and 175087. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: pekmezovic@sezampro.rs

These authors equally contributed to this work.

Introduction

Bladder cancer is the second most common malignancy of the urinary tract and has the second highest mortality rate among urological neoplasms [1]. It affected 73,510 patients and lead to 14,880 deaths in 2012 worldwide [2]. Demographic characteristics associated with the greatest risk for bladder cancer include male gender, white race and the increasing age [3]. It is generally estimated that the male:female incidence ratio is 3.8:1.0 [3]. The most frequent pathohistological type of bladder cancer is urothelial carcinoma, also called transitional cell carcinoma (TCC), accounting for approximately 90% of all bladder cancers [3]. It has been known that uroepithelial cells are most vulnerable to metabolic end products of different compounds, including carcinogens. This
malignancy is characterized by multifactorial etiology, involving both genetic and environmental factors.

The well established risk factors for bladder cancer include cigarette smoking (50% cases in men, 30% cases in women), but also exposure to occupational agents [3]. Occupational exposures account for 5 to 25% of all bladder cancer cases [4]. Over 40 occupations have been associated with an elevated risk of bladder cancer in epidemiologic studies, but the evidence is compelling for only a few. Those established at risk industries include the manufacturing of products such as synthetic dyes and paints, cables, textiles, leather works, and aluminum and the petrochemical, coal tar, and rubber industries [5,6]. A number of specific occupations have also been identified to be associated with increased risk of bladder cancer. These include, but are not limited to, cooks and kitchen workers, electricians, hairdressers, leather workers, machinists, petroleum workers, rubber workers, cosmetiners, truckers, and vehicle mechanics, as summarized by Schulte et al. [7] in 1987, as well as coke oven workers, roofers, dry cleaners, chimney sweeps, and painters, as addressed by others in more recent literature [5–8,10].

Despite the fact that occupations associated with bladder cancer have been well established, the question still arises why individuals with seemingly equal exposure to occupational carcinogens develop bladder cancer in an unpredictable manner. This is probably attributed to genetic polymorphisms of the genes coding for the xenobiotic metabolizing enzymes, particularly glutathione S-transferase (GST). GSTs catalyze the conjugation of glutathione on electrophilic substrates and are an important line of defense in the protection of cellular components against reactive species. The most well characterized GST genotype classes have been named alpha (GSTA), mu (GSTM), pi (GSTP) and theta (GSTT). Appreciable GST activities are seen in bladder epithelium [11]. GST enzymes that belong to various classes have different, but sometimes overlapping, substrate specificities. Several types of allelic variations have been identified within GST classes, with that in the GSTM1, GSTT1 and GSTP1 genes receiving the most attention in genetic epidemiological studies [12]. Individuals homozygous for the GSTM1*0 and GSTT1*0 alleles (frequently referred to as GSTM1-null and GSTT1-null genotypes), which comprise for 50% and 11–18% of white population, respectively [13,14], exhibit loss of GSTM1 and GSTT1 enzymatic activity. Single-nucleotide polymorphism (SNP) leading to amino acid substitution from isoleucine (Ile) to valine (Val) changes catalytic activity of the GSTP1 enzyme [15]. In healthy Caucasians, the frequencies of the genotype variants of GSTP Ile/Ile, Ile/Val and Val/Val are 51.5, 39.4, and 9.1%, respectively [15]. The role of GSTM1 polymorphism has emerged relatively recently in genetic epidemiological studies. It is represented by three, apparently linked, single nucleotide polymorphisms (SNPs): -567T/TG, -69COT, -52GOA [16]. These substitutions result in differential expression of lower transcriptional activation of variant GSTM1*B (-567G, -69T, -52A) than common GSTM1*A allele (-567T, -69C, -52G) [16]. The relative frequencies of GSTA1-A1, AB and BB genotype in Caucasians are 38%, 48% and 14%, respectively [16].

DNA extraction and genotyping

Genomic DNA was isolated from whole blood using the QIAGEN QI Amp (Qiagen, Inc., Chatsworth, CA, USA) 96-spin blood protocol according to the manufacturer’s instructions. Blood was collected when patients were admitted to the clinic. GSTM1 genotyping was performed by multiplex PCR method [17]. Primers used were GSTM1 forward: 5’-GAACCTCCCT-
amplification 176 bp products (20
Primers used were
GSTT1- forward: 5'GGTCCTCACATCTC-3' and reverse: 5'-ACCCCAGGGCTCTATGGGAA-3', indicated mutant allele (Val/Val), while if CYP1A1 polymorphism incurred, it resulted in one more fragment of 481 bp.

All genotyping was performed by laboratory personnel blinded to case-control status, and blinded quality control samples were inserted to validate genotyping identification procedures; concordance for blinded samples was 100%.

Statistical analysis
The distribution of the GSTA1 and GSTP1 polymorphisms for the case and control populations was tested for the Hardy–Weinberg equilibrium by χ² test. As a measure of effect size, odds ratio (OR) with corresponding 95% confidence interval (95%CI) was used to describe the strength of association between the genotypes and bladder cancer modified by occupational exposure. Unconditional logistic regression analysis is applied. Bearing in mind that age and smoking are well established risk factors for bladder cancer, we adjusted OR by these variables as potential confounders. Interactions between GST polymorphisms and occupational exposure were included in the logistic regression models and also adjusted by potential confounding variables. The probability level of ≤0.05 was considered statistically significant. For statistical analysis the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA.) was used.

Results
Table 1 shows selected characteristics of male patients with bladder cancer and their controls. The smoking prevalence among cases was higher (82%) than the prevalence found in controls (66%) with the smokers being at 2.3-fold higher risk for TCC than non-smokers (95% CI = 1.3–4.1, p = 0.005). Furthermore, occupationally exposed men had 3.2 times higher risk for TCC than those unexposed (95% CI = 1.6–6.6, p = 0.001). We observed the significantly higher risk in those men occupationally exposed to organic solvents (OR = 3.4, 95% CI = 1.5–7.3, p = 0.002).

Genotyping was conducted for all recruited patients (Table 2). The GSTA1 and GSTP1 genotype frequencies were in Hardy-Weinberg equilibrium both for cases and controls (p>0.05). The
observed genotype frequencies in controls were not significantly different from frequencies previously described among Caucasians. However, the frequency of GSTT1-null genotype in control group (20%) was higher than values reported among Caucasians (18.1%). As shown in Table 2, the frequencies of GST null/low-activity genotypes were higher in cases compared to controls with the exception of the GSTT1-null genotype. Although GSTA1, T1 and P1 genotypes did not contribute independently toward the risk of TCC, the GSTM1-null genotype was overrepresented among cases (56%) compared to M1-active genotype with an adjusted OR of 2.1 reaching a statistical significance (95% CI = 1.1–4.2, p = 0.032).

Combined effects of GSTA1, GSTM1, GSTP1 and GSTT1 polymorphisms and occupational exposure on bladder cancer risk in male patients are shown in Table 3. When both cases and controls were dichotomized according to both genotype and occupational exposure, exposed subgroup was at TCC risk regardless of GST genotype. We found that occupationally exposed individuals with GSTT1-active genotype exhibited 4.3-fold increased risk compared to the unexposed T1-active subjects (95% CI = 1.7–10.6, p = 0.002). However, only for the GSTP1 gene is there evidence of a gene–occupational exposure interaction (p = 0.017).

In order to test whether GST-occupational exposure interaction is modified by the specific type of exposure, cases and controls were further stratified into exposed to solvents and exposed to pesticides. Combined effect of occupational exposure to solvents and GST genotype on bladder cancer risk in male patients is shown on Table 4. The results point to the importance of antioxidant GSTA1 and GSTM1 activity protection against free radicals produced during solvent metabolism. The risk of TCC development was 3.5-fold elevated among GSTT1-active patients exposed to solvents in comparison with GSTT1-unexposed patients (95% CI = 1.9–15.1, p = 0.002). Significant association was also found for GSTP1 Ile/Ile individuals who had 3.3 higher TCC risk compared to the unexposed Ile/Ile individuals (95% CI = 1.0–10.8, p = 0.047). However, only for GSTP1 statistically significant interaction between genotype and occupational exposure to solvents was found (p = 0.044).

Combined effect of occupational exposure to pesticides and GST genotype on bladder cancer risk in male patients is shown on Table 5. Men with GSTT1-active genotype exposed to pesticides exhibited 4.5 times higher risk in comparison with unexposed GSTT1-active subjects (95% CI = 0.9–22.5, p = 0.067).

**Discussion**

Our results showed that occupationally exposed men had 3 times higher risk for TCC. This result confirms the occupational exposure as a TCC risk factor [4]. Furthermore, analysis of gene–occupational exposure interaction indicated a significant effect between occupationally exposed men and GSTP1 polymorphism. GSTP1 seems to play a role of particular importance in the detoxification of inhaled toxicants in occupationally exposed individuals since it is the most abundant GST isoform in the lung [19]. The mutated GSTP1 seems to be less effective in detoxification than the wild genotype [20]. Thus, Heuser et al. [18] showed that the mutated genotype (Ile/Val or Val/Val) was

### Table 2. GSTA1, GSTM1, GSTT1 and GSTP1 genotypes in relation to bladder cancer risk in male patients.

| GST genotype | Cases | Controls | OR (95% CI) | p |
|--------------|-------|----------|-------------|---|
| **GSTA1**    |       |          |             |   |
| AA           | 45 (31)| 41 (36)  | 1.0 (reference group) | |
| AB           | 81 (57)| 54 (47)  | 1.9 (0.9–4.2) | 0.094 |
| BB           | 17 (12)| 19 (17)  | 1.1 (0.4–2.9) | 0.875 |
| AB+BB        | 98 (69)| 73 (64)  | 1.7 (0.8–3.5) | 0.171 |
| **GSTM1**    |       |          |             |   |
| active*      | 63 (44)| 58 (51)  | 1.0 (reference group) | |
| null*        | 80 (56)| 56 (49)  | 2.1 (1.1–4.2) | 0.032 |
| **GSTT1**    |       |          |             |   |
| active*      | 101 (74)| 82 (72) | 1.0 (reference group) | |
| null*        | 36 (26)| 32 (28)  | 1.0 (0.5–2.2) | 0.999 |
| **GSTP1**    |       |          |             |   |
| Ile/Ile      | 62 (43)| 49 (43)  | 1.0 (reference group) | |
| Ile/Val      | 65 (46)| 48 (42)  | 0.92 (0.5–1.9) | 0.918 |
| Val/Val      | 16 (11)| 17 (15)  | 0.6 (0.2–1.9) | 0.401 |
| Ile/Val+Val/Val | 81 (57)| 65 (47) | 0.9 (0.4–1.7) | 0.876 |

*Active (present) if at least one active allele present.

Inactive (null) if no active alleles present. OR- odds ratio adjusted for age and pack-years. CI- confidence interval.

doi:10.1371/journal.pone.0099448.t002
associated with greater DNA damage in Brazilian footwear workers than the wild (Ile/Ile) genotype [21]. These studies point to an interaction between the exposure and GSTP1 genotype. In our study, the most significant TCC risk was found for solvents. Epidemiologic evidence on the relationship between solvents and various cancers, such as gastrointestinal cancers, lung cancer and lymphohematopoietic malignancies, is well established [22]. Among compounds that have carcinogenic role halogenic aliphatic solvents have been mostly described. There are few reports about relationship between urinary bladder risk and solvents. Previous case-control studies reported significantly increased risks (between 3.1 and 8.8 times) among workers in the dyestuffs industry [23,24]. Several other investigators have reported elevated risks for spray painters [25,26], who have been reported to be exposed to many known or suspected carcinogens, including solvents. On the other hand, Lohi and others [27] found that among Finnish workers exposure to solvents was positively associated with the incidence of bladder cancer in women, but not in men.

It is important to note that risk imposed by occupational hazards was modified by GST polymorphism. We observed that individuals occupationally exposed to solvents with at least one low activity GSTA1 allele had the highest risk (about 9 times), while GSTM1-null carriers had 6.5 times higher bladder cancer risk when compared to unexposed GSTA1 AA and GSTM1-active persons, respectively. This result was expected since in several malignant diseases, such as colorectal, prostate and hepatocellular cancer, GSTA1*B allele with lower transcriptional activity was associated with increased risk. GSTA1 protein belongs to the most promiscuous GSTs that acts upon a broad range of substrates which bind to its active site [28]. Our findings that low-activity GSTA1 and GSTM1-null genotype increase susceptibility to bladder cancer in occupationally exposed men can be explained by the role of GST enzymes in detoxification and in antioxidant defense. Namely, GSTA1 and GSTM1 possess strong peroxidase activity and are key components in cellular defense against free radicals [29]. It may be speculated that free radicals are produced during solvent metabolism [30]. Regarding potential place of solvent detoxification, it is important to note that uroepithelial cells do not express GSTA1, while their GSTM1 protein level is also relatively low [31]. On the other hand, liver cells abundantly express GSTA1 and GSTM1 and thus participate in GSTA1 and GSTM1 mediated conjugation of different metabolites with glutathione, thereby enhancing their excretion in urine [32]. Taken together, these data suggest that liver, by its GSTs conjugating and peroxidase activity plays a key role in protection against bladder carcinogens present in halogenated solvents. On the other hand, GSTT1-active individuals occupationally exposed to solvents exhibited 5 times higher risk of TCC in comparison with GSTT1-active unexposed subjects. These results are biologically

### Table 3. Combined effect of occupational exposure and GST genotype on bladder cancer risk in male male patients.

| GST/exposure | Cases | Controls | OR (95%CI) | p |
|--------------|-------|----------|------------|---|
|              | n (%) | n (%)    |            |    |
| GSTA1        |       |          |            |    |
| AA/unexposed | 21 (15%) | 32 (28%) | 1.0 (reference group) |  |
| AA+BB/unexposed | 56 (39%) | 48 (42%) | 2.4 (0.8–7.3) | 0.121 |
| AA/exposed | 24 (17%) | 9 (8%) | 6.2 (1.4–27.1) | 0.015 |
| AA+BB/exposed | 42 (29%) | 25 (22%) | 6.4 (2.0–20.2) | 0.002 |
| P interaction between genotype and occupational exposure | = 0.104 |
| GSTM1        |       |          |            |    |
| active*/unexposed | 35 (24%) | 44 (39%) | 1.0 (reference group) |  |
| null*/unexposed | 42 (29%) | 36 (32%) | 3.3 (1.2–9.4) | 0.023 |
| active/exposed | 28 (20%) | 14 (12%) | 5.4 (1.9–15.8) | 0.002 |
| null/exposed | 38 (27%) | 20 (17%) | 6.0 (2.2–16.5) | 0.001 |
| P interaction between genotype and occupational exposure | = 0.601 |
| GSTT1        |       |          |            |    |
| active*/unexposed | 54 (40%) | 57 (50%) | 1.0 (reference group) |  |
| null*/unexposed | 22 (16%) | 23 (20%) | 1.3 (0.5–3.9) | 0.577 |
| active/exposed | 47 (34%) | 25 (22%) | 4.3 (1.7–10.6) | 0.002 |
| null/exposed | 14 (10%) | 9 (8%) | 2.6 (0.8–8.9) | 0.124 |
| P interaction between genotype and occupational exposure | = 0.770 |
| GSTP1        |       |          |            |    |
| Ile/Ile/unexposed | 31 (22%) | 32 (28%) | 1.0 (reference group) |  |
| Ile/Val+Val/Val/unexposed | 46 (32%) | 48 (42%) | 0.8 (0.3–2.1) | 0.605 |
| Ile/Ile/exposed | 31 (22%) | 17 (15%) | 2.8 (1.0–7.9) | 0.049 |
| Ile/Val+Val/Val/exposed | 35 (24%) | 17 (15%) | 2.8 (1.0–8.0) | 0.049 |
| P interaction between genotype and occupational exposure | = 0.017 |

*aActive (present) if at least one active allele present.*  
*bInactive (null) if no active alleles present.*  
*OR- odds ratio adjusted for age and pack-years. CI- confidence interval.*  
*doi:10.1371/journal.pone.0099448.t003*
plausible since GST-mediated conjugation with halogenated substrates may lead to a more toxic or mutagenic metabolites. Namely, substrates with ≥2 halogenes are activated because the conjugated product is instable, leading to reactions with nucleophiles, particularly DNA and proteins [33]. The human polymorphic GSTT1 catalyze conjugation of halomethanes, dihalomethanes, ethylene oxide and a number of other industrial compounds. Our results confirm the assumption of Avima M Ruder et al. [34] that humans with fully functional GST genes produce enzymes that metabolize some solvents to cytotoxic metabolites; while those with less functional or nonfunctioning genes have little or no enzyme and apparently do not produce cytotoxic metabolites from solvent exposure. Until now, the association between GST polymorphism and occupationally related cancers has been studied mostly in renal cell carcinoma. Results of these studies showed that GSTT1-active genotype enhanced the risk of renal cell carcinoma among subjects exposed to solvents. Our results on higher bladder carcinoma risk in GSTT1-active individuals occupationaly exposed to solvents are in accordance with previously published results in renal cell carcinoma [35,36]. Regarding the potential mechanism of solvent metabolism by GST, it is generally assumed that the main site is liver, followed by a mandatory transfer of conjugates to the kidney. However, the initial bioactivation step of halogenated solvents, can take place in the kidney itself [37]. Uroepithelium is also capable of metabolizing some procarcinogens to inactive or genotoxic metabolites, and is, therefore, not exposed only to preformed reactive metabolites in the urine [38]. As the renal parenchyma and uroepithelium are exposed to the same broad range of potentially genotoxic compounds, the potential genotoxicity of carcinogens also depends on the biotransformation capacity of these tissues. As a result of GST polymorphism, great interindividual differences in GST isoenzyme profiles exist, in both renal parenchyma and uroepithelial cells [37].

Table 4. Combined effect of occupational exposure to solvents and GST genotype on bladder cancer risk in male patients.

| GST genotype | Cases | Controls | OR (95% CI) | P |
|--------------|-------|----------|-------------|---|
|              | n (%) | n (%)    |             |    |
| GSTA1        |       |          |             |    |
| AA/unexposed | 21 (1%)| 32 (32%) | 1.0 (reference group) |  |
| AB+BB/unexposed | 56 (46%)| 48 (49%) | 2.4 (0.8–7.3) | 0.121 |
| AA/solvents  | 14 (11%)| 6 (6%)   | 5.9 (1.0–33.1) | 0.046 |
| AB+BB/solvents | 31 (25%)| 13 (13%) | 9.2 (2.4–34.7) | 0.001 |
| P interaction between genotype and occupational exposure to solvents = 0.228 |
| GSTM1        |       |          |             |    |
| active+/unexposed | 35 (28%)| 44 (43%) | 1.0 (reference group) | 0.023 |
| null+/unexposed | 42 (34%)| 36 (35%) | 3.3 (1.2–9.4) | 0.006 |
| active+/solvents | 21 (17%)| 10 (10%) | 4.7 (1.6–13.8) | 0.001 |
| null+/solvents | 27 (22%)| 12 (12%) | 6.5 (2.1–19.7) | 0.001 |
| P interaction between genotype and occupational exposure to solvents = 0.896 |
| GSTT1        |       |          |             |    |
| active+/unexposed | 54 (46%)| 57 (56%) | 1.0 (reference group) | 0.047 |
| null+/unexposed | 22 (18%)| 23 (22%) | 1.3 (0.5–3.9) | 0.577 |
| active+/solvents | 34 (29%)| 15 (15%) | 5.3 (1.9–15.1) | 0.002 |
| null+/solvents | 8 (7%) | 7 (7%)   | 1.7 (0.4–7.3) | 0.470 |
| P interaction between genotype and occupational exposure to solvents = 0.224 |
| GSTP1        |       |          |             |    |
| Ile/Ile/unexposed | 31 (25%)| 32 (31%) | 1.0 (reference group) | 0.605 |
| Ile/Val+Val/Val/unexposed | 46 (37%)| 48 (47%) | 0.8 (0.3–2.1) | 0.047 |
| Ile/Ile/solvents | 22 (18%)| 9 (9%)   | 3.3 (1.0–10.8) | 0.089 |
| Ile/Val+Val/Val/solvents | 26 (21%)| 13 (13%) | 2.6 (0.9–7.9) | 0.089 |
| P interaction between genotype and total occupational exposure to solvents = 0.044 |

*a* Active (present) if at least one active allele present.

*b* Inactive (null) if no active alleles present. OR: odds ratio adjusted for age and pack years; CI: confidence interval.

doi:10.1371/journal.pone.0099448.t004
to risk of carcinoma of urinary tract. Namely, Karami and others reported that renal cell carcinoma risk associated with pesticide exposure was highest among individuals with active GSTM1/T1 genotypes [44]. Although we did not observe significant effect between exposure to pesticides and GST polymorphisms we found borderline significance for GSTT1-active genotype. One of the reasons for non-significant association between GSTT1-active genotype may be the relatively small number of pesticide exposed participants in both case and control groups. Nevertheless, it is well known that pesticides produced from halogenated alkanes, alkenes undergo bioactivation in the liver and kidney after conjugation to glutathione by GSTT1 [41]. Therefore, an active GSTT1 enzyme will be required to conjugate substrates and form more reactive intermediates that directly damage tissues. Conversely, the deleted variant of GSTT1-genotype will form an inactive enzyme and therefore metabolism of halogenated compounds will occur through oxidation, without formation of reactive intermediates [44].

The principal limitations of this study are the relatively small sample size which limiting the precision of the odds ratios, hospital-based control group and qualitative evaluation of occupational exposure. Concerning the actual sample size (143 cases and 114 controls), the statistical power is 66%. Furthermore, it is well known that relatively small numbers of both study participants and GST polymorphisms studied might be sources of potential biases which may influence the study findings. However, we tested effects of four GST polymorphisms and occupational exposure on bladder cancer risk and therefore significantly decreased chance for publication bias. Additionally, we cannot entirely rule out the possibility that some of our results could be caused by confounding, although we included only men and adjusted all results by age and smoking status. Further studies with larger samples and more rigorous designs are needed to investigate the gene effects and the potential effect modification by environmental factors.

Conclusions

GSTM1-null genotype increased the risk of bladder cancer in males. Null or low-activity genotypes of the GSTA1, GSTT1, and GSTP1 did not contribute independently towards the risk of bladder cancer in males. However, in association with occupational exposure, both low activity GSTA1 and GSTM1-null genotype increase individual susceptibility to bladder cancer suggesting the protective role of these detoxification and antioxidant enzymes in metabolism of occupational hazards, specifically organic solvents. On the other hand, the presence of GSTT1-active genotype in occupationally exposed subjects, resulting in GSTT1 protein expression and GSTT1 mediated bioactivation, increases the risk of bladder cancer.

Table 5. Combined effect of occupational exposure to pesticides and GST genotype on bladder cancer risk in male patients.

| GST/exposure   | Cases          | Controls        | OR (95%CI) | p   |
|---------------|----------------|-----------------|------------|-----|
|               | n (%)          | n (%)           |            |     |
| GSTA1         |                |                 |            |     |
| AA/unexposed  | 21 (22%)       | 32 (36%)        | 1.0 (ref)  |     |
| AB+BB/unexposed | 56 (60%)  | 48 (54%)        | 2.4 (0.8–7.3) | 0.121|
| AA/pesticides | 8 (9%)         | 3 (3%)          | 4.2 (0.5–36.0) | 0.190|
| AB+BB/pesticides | 8 (9%)   | 6 (7%)          | 2.0 (0.5–7.9) | 0.239|
| P interaction between genotype and occupational exposure to pesticides = 0.957 |

| GSTM1          |                |                 |            |     |
|               |                |                 |            |     |
| active*/unexposed | 35 (37%)  | 44 (49%)        | 1.0 (ref)  |     |
| null*/unexposed | 42 (45%)       | 36 (41%)        | 3.3 (1.2–9.4) | 0.023|
| active/pesticides | 7 (8%)      | 3 (3%)          | 2.9 (0.7–12.2) | 0.138|
| null/pesticides  | 9 (10%)        | 6 (7%)          | 1.9 (0.5–6.7) | 0.264|
| P interaction between genotype and occupational exposure to pesticides = 0.125 |

| GSTT1          |                |                 |            |     |
|               |                |                 |            |     |
| active*/unexposed | 54 (59%)  | 57 (64%)        | 1.0 (ref)  |     |
| null*/unexposed | 22 (24%)       | 23 (25%)        | 1.3 (0.5–3.9) | 0.577|
| active/pesticides | 11 (12%)   | 7 (8%)          | 4.5 (0.9–22.5) | 0.067|
| null/pesticides  | 5 (5%)         | 2 (3%)          | 2.6 (0.4–20.6) | 0.264|
| P interaction between genotype and occupational exposure to pesticides = 0.508 |

| GSTP1          |                |                 |            |     |
|               |                |                 |            |     |
| Ile/Ile/unexposed | 31 (33%)  | 32 (36%)        | 1.0 (ref)  |     |
| Ile/Val+Val/Val/unexposed | 46 (49%) | 48 (53%)      | 0.8 (0.3–2.1) | 0.605|
| Ile/Ile/pesticides | 9 (10%)     | 6 (7%)          | 2.9 (0.6–13.6) | 0.181|
| Ile/Val+Val/Val/pesticides | 7 (8%)  | 3 (4%)          | 2.4 (0.5–10.1) | 0.231|
| P interaction between genotype and occupational exposure to pesticides = 0.320 |

*Active (present) if at least one active allele present.

Inactive (null) if no active alleles present. OR- odds ratio adjusted for age and pack years. CI- confidence interval.

doi:10.1371/journal.pone.0099448.005
Acknowledgments
The authors would like to thank technician Miss Sanja Zivotic for collecting data and support in manuscript preparation as well as Professor Goran Trajkovic, for final statistical consultancy.

Author Contributions
Conceived and designed the experiments: MGM VMC TPS TDP. Performed the experiments: MGM VMC TDP. Analyzed the data: MGM VMC ARSR MSPE TPS TDP. Contributed reagents/materials/analysis tools: PVB DPD. Wrote the paper: MGM VMC ARSR MSPE TPS TDP.

References
1. Kim JJ (2012) Recent advances in treatment of advanced urothelial carcinoma. Curr Urol Rep 13:147–52.
2. Siegel R, Naishadham D, Jemal A (2012) Cancer statistics. CA Cancer J Clin 62:10–29.
3. American Cancer Society (2012) Bladder Cancer 2012. American Cancer Society, Atlanta, USA.
4. Offen SM, Elkoubsy SA, DelCelso GL (2006) An updated review of the literature: risk factors for bladder cancer with focus on occupational exposures. South Med J 99:1256–63.
5. Clapp RW, Howe G, Lefever MJ (2003) Environmental and occupational causes of cancer. A Review of Recent Scientific Literature. Lowell Center for Sustainable Production. Low Mass, USA.
6. International Agency for Research on Cancer (2010) Painting, fire-fighting, and shiftwork. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. pp. 43–394.
7. Schulte PA, Rennert G, Demoitie J, Ward E (1987) Occupational cancer of the urinary tract. Occup Med 2: 85–107.
8. International Agency for Research on Cancer (2010) Some aromatic amines, organic dyes, and related exposures. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. pp. 1–692.
9. International Agency for Research on Cancer (2010) Some non-heterocyclic polycyclic aromatic hydrocarbons and some related compounds. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. pp. 754–759.
10. Pukalik E, Martens JI, Lyne G, Gunnarsdottr IJK, Sparéi P, et al. (2009) Occupation and cancer follow-up of 15 million people in free Nordic countries. Acta Oncologica 48: 640–780.
11. Simic T, Mimic-Oka J, Savic-Radojevic A, Matic M, Savic-Radojevic A, et al. (2009) Glutathione S-transferase T1-1 activity upregulated in transitional cell carcinoma of urinary bladder. Urology 65: 1035–40.
12. Di Pietro G, Magnin LA, Rios-Santos F (2010) Glutathione S-transferases: an overview in cancer research. Expert Opin Drug Metab Toxicol 6: 153–70.
13. Eaton DL, Bamberl TK (1999) Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol Sci 49: 156–64.
14. Audh S (2000) Mammalian class θ GST and differential susceptibility to carcinogens: a review. Mutat Res 463: 247–83.
15. Watson MA, Stewart RK, Smith GB Massey TE, Bell DA (1998) Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 19: 273–80.
16. Coles FB, Kadlubow FF (2005) Human alpha class glutathione S-transferases: genetic polymorphism, expression, and susceptibility to disease. In: Helmut S, Lester P, editors. Glutathione Transferases and Gamma-Glutamyl Transpeptidases, Methods Enzymology. London: Elsevier Academic Press. pp 9–42.
17. Abdel-Rahman SZ, El-Zein RA, Arif F (2006) Glutathione S-transferases M1-1 and T1-1 as risk modifiers for renal cell cancer associated with occupational exposure to chemicals. Occup Environ Med 66: 789–93.
18. Buzio J, De Palma G, Mouzoni P, Tondel M, Buzio C, et al. (2003) Glutathione S-transferase-P1 expression correlates with increased antioxidant capacity in transitional cell carcinoma of the urinary bladder. Eur Urol 52: 470–7.
19. Ruder AM, Guarnieri C, Rosetti S, Gobba F, Ghissotti S, et al. (1999) Genetic polymorphisms influence variability in benzene metabolism in humans. Pharmacogenetics 9: 445–51.
20. Landi S (2000) Mammalian class θ GST and differential susceptibility to carcinogens: a review. Toxicol Lett 127: 321–7.
21. Savic-Radojevic A, Mimic-Oka J, Pjesa-Ercegovac M, Opacic M, Dragicevic D, et al. (2007) Glutathione S-transferase-P1 expression correlates with increased antioxidant capacity in transitional cell carcinoma of the urinary bladder. Eur Urol 52: 470–7.
22. Clapp RW, Howe G, Lefever MJ (2003) Activation of alkyl halides by glutathione transferases. In: Helmut S, Lester P, editors. Glutathione Transferases and Gamma-Glutamyl Transpeptidases, Methods Enzymology. London: Elsevier Academic Press pp 9–42.
23. Band PR, Le ND, MacArthur AC, Fang R, Gallagher RP (2005) Identification of occupational cancer risks in British Columbia: a population-based case-control study of 1129 cases of bladder cancer. J Occup Environ Med 47: 854–8.
24. Buzio J, De Palma G, Mouzoni P, Tondel M, Buzio C, et al. (2003) Glutathione S-transferase-P1 expression correlates with increased antioxidant capacity in transitional cell carcinoma of the urinary bladder. Eur Urol 52: 470–7.
25. La Vecchia C, Negri E, D'Avanzo B, Franceschi S (1990) Occupation and the risk of bladder cancer. Int J Epidemiol 19: 264–8.
26. Corrêa S, Clavel J, Límaco JC, Boccon-Gibod L, Le Moulé N, et al. (1993) Occupational risks of bladder cancer in France: A multicentre case-control study. Int J Epidemiol 22: 402–11.
27. Lohi J, Kyyrönen P, Kauppinen T, Kujala V, Pukalik E (2008) Occupational exposure to solvents and gasoline and risk of cancers in the urinary tract among Finnish workers. Am J Ind Med 51: 668–72.
28. Vonakis S, Merlo P, Pearce N, Puntoni R (1989) Bladder cancer: an occupational exposure to polycyclic aromatic hydrocarbons. Int Cancer 44: 648–651.
29. La Vecchia C, Negri E, D’Avanzo B, Franceschi S (1990) Occupation and the risk of bladder cancer. Int J Epidemiol 19: 264–8.
30. Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR (1997) Association between glutathione S-transferase P1 gene polymorphism and younger age at onset of lung carcinoma. Cancer 107: 1570–7.
31. Savic-Radojevic A, Mimic-Oka J, Pljesa-Ercegovac M, Opacic M, Dragicevic D, et al. (2007) Glutathione S-transferase-P1 expression correlates with increased antioxidant capacity in transitional cell carcinoma of the urinary bladder. Eur Urol 52: 470–7.
32. Ruder AM, Guarnieri C, Rosetti S, Gobba F, Ghissotti S, et al. (1999) Genetic polymorphisms influence variability in benzene metabolism in humans. Pharmacogenetics 9: 445–51.
33. Clapp RW, Howe G, Lefever MJ (2003) Activation of alkyl halides by glutathione transferases. In: Helmut S, Lester P, editors. Glutathione Transferases and Gamma-Glutamyl Transferases, Methods Enzymology. London: Elsevier Academic Press pp 9–42.
34. Band PR, Le ND, MacArthur AC, Fang R, Gallagher RP (2005) Identification of occupational cancer risks in British Columbia: a population-based case-control study of 1129 cases of bladder cancer. J Occup Environ Med 47: 854–8.
35. Zahm SH (1997) Mortality study of pesticide applicators and other employees of a lawn care service company. J Occup Environ Med 39: 1055–67.
36. Buzio J, De Palma G, Mouzoni P, Tondel M, Buzio C, et al. (2003) Glutathione S-transferase-P1 expression correlates with increased antioxidant capacity in transitional cell carcinoma of the urinary bladder. Eur Urol 52: 470–7.
37. Simic T, Savic-Radojevic A, Pjesa-Ercegovac M, Matic M, Mimic-Oka J (2009) Glutathione S-transferases in kidney and urinary bladder tumors. Nat Rev Urol 6: 281–9.
38. Thier R, Golka K, Bürning T, Ko Y, Bolt HM (2002) Genetic susceptibility to environmental toxicants: the interface between human and experimental studies in the development of new toxicological concepts. Toxicol Lett 127: 321–7.
39. Band PR, Le ND, MacArthur AC, Fang R, Gallagher RP (2005) Identification of occupational cancer risks in British Columbia: a population-based case-control study of 1129 cases of bladder cancer. J Occup Environ Med 47: 854–8.
40. Zahm SH (1997) Mortality study of pesticide applicators and other employees of a lawn care service company. J Occup Environ Med 39: 1055–67.
41. Meier F, Faller GC, Hirvonen A, Falck G, Norppa H (1996) Cytogenetic monitoring of occupational exposure to pesticides: characterization of GSTM1, GSTT1, and NAT2 genes in pesticide-exposed greenhouse workers. Mutat Res 414: 225–37.
42. Falck GC, Hirvonen A, Scarpato R, Szamoksi ST, Migliore L, et al. (1999) Micronuclei in blood lymphocytes and genetic polymorphism for GSTM1, GSTT1 and NAT2 in pesticide-exposed greenhouse workers. Mutat Res 441: 205–19.
43. Scarpato R, Migliore L, Hirvonen A, Falck G, Norppa H (1996) Cytogenetic monitoring of occupational exposure to pesticides: characterization of GSTM1, GSTT1, and NAT2 genes. Environ Mol Mutagen 27: 263–9.
44. Carami S, Boffetta P, Rothman N, Hung RJ, Stewari T, et al. (2008) Renal cell carcinoma, occupational pesticide exposure and modification by glutathione S transferase polymorphisms. Carcinogenesis 29: 1567–71.
45. Bonassi S, Merlo P, Pearce N, Puntoni R (1989) Bladder cancer: an occupational exposure to polycyclic aromatic hydrocarbons. Int Cancer 44: 648–651.