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*Original Article*

# Association between *GSTM1* copy number, promoter variants and susceptibility to urinary bladder cancer

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**Abstract:** This study sought to determine the role of copy number variants (CNV) combined with other genetic variants in the Glutathione *S*-transferases Mu class1 (*GSTM1)* promoter in the development of urinary bladder cancer. TaqMan real-time PCR and direct sequencing were used to determine genetic variants. Haploblocks and haplotype were constructed and estimated by Haploview and Phase, respectively. Logistic regression revealed a significantly decreased bladder cancer risk in subjects with at least 2 copies of *GSTM1* (OR=0.56; 95%CI=0.39-0.81) but not in those with 1 copy of the gene. *GSTM1* promoter screening revealed an insertion variant (-1543TTCT) and 14 single nucleotide polymorphisms (SNPs) (-1529C>G, -1490A>G, -1143A>G, -888A>T, -498G>C, -486C>G, -471C>T, -426G>A, -344C>T, -343A>T, -341C>T, -339C>T, -304G>A, and -164C>T). Four haploblocks were evident by Haploview. There was no significant association between any single SNP/haplotype and bladder cancer risk. However, when stratified by copy number, the two copy carriers with the -1543 insertion had decreased bladder cancer risk (OR, 0.58; 95%CI, 0.32-0.10) and similar results were found in two copy carriers with -888 A, -486G, - 344 C, -343 A, -341 C allele and haplotype $INS_{-1543}$-$C_{-1529}$-$A_{-1429}$ in LD block 1, $A_{-1143}$-$A_{-888}$ in LD block 2, $C_{-498}$-$G_{-486}$-$T_{-471}$ in LD block 3, $C_{-344}$-$A_{-343}$-$C_{-341}$-$C_{-339}$ and $C_{-344}$-$A_{-343}$-$C_{-341}$-$T_{-339}$ in LD block 4. These results suggest that *GSTM1* CNV is a better predictor of bladder cancer susceptibility than measuring presence/absence of *GSTM1* and other genetic variants also can modify bladder cancer risk.

**Keywords:** Glutathione-S-transferase M1, Copy number variant, single nucleotide polymorphism, bladder cancer

## Introduction

Glutathione *S*-transferases (GSTs) catalyze the conjugation of the cellular tripeptide glutathione (GSH) with a number of electrophilic compounds, including chemical carcinogens, environmental pollutants, and anticancer drugs [1]. Usually, the electrophilic chemicals are rendered less toxic by conjugation with glutathione. Consequently, the expression level of GSTs is a crucial factor in defending cells against a broad spectrum of xenobiotic toxins, and is an important determinant of antineoplastic drug metabolism [2, 3]. Two major families of GST proteins, cytosolic and membrane-bound GSTs, are involved in Phase II biotransformation processes. Cytosolic GST enzymes, the largest subfamily of this superfamily, are encoded by at least five gene families (class alpha, mu, pi, sigma, and theta GST), based on sequence homologies and other common properties. Among these, the class alpha, mu and pi enzymes are the most abundant GSTs. The membrane-bound GST proteins comprise microsomal GSTs and membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEGs) related to the synthesis of prostaglandins and leukotrienes [4, 5]. Different GST subunits have variable tissue distribution and substrate specificities; however, most tissues have specific isoenzyme expression patterns. For instance, the liver, testis, brain, and urinary bladder have the highest source of GSTM1, but significant amounts are also found in colon and pancreas. In contrast, GSTM3 is expressed at relatively high levels in the lungs and upper respiratory tract, although GSTP1 is the dominant isoform [6]. To date, at least 16 different GST enzymes have been identified in humans [7].

Located in a 20kb GST mu gene cluster on chromosome 1p13.3 [8], human *GSTM1* has com-

**Table 1.** Distribution of select characteristics among patients and controls.

| Variables | No. of patients (%) | No. of controls (%) | *P* value |
|---|---|---|---|
| Total no. | 710 (100%) | 782 (100) | |
| Gender | | | 0.004 |
| Male | 552 (77.75) | 557 (71.23) | |
| Female | 158 (22.25) | 225 (28.77) | |
| Age, mean (SD) | 64.24 (11.31) | 63.64 (11.84) | 0.32 |
| Median (Range) | 66 (21-89) | 64 (21-94) | |
| Smoking Status | | | <0.001 |
| Never smoker | 190 (26.76) | 366 (46.80) | |
| Former smoker | 330 (46.48) | 350 (44.76) | |
| Current smoker | 190 (26.76) | 66 (8.44) | |
| Pack-year, median (range) | 38 (0-176) | 20 (0-165) | <0.001 |

mon variants including copy number variants and single nucleotide polymorphisms (SNPs) [6]. The homozygous deletion (null variant) of the *GSTM1* gene has been observed in different ethnic populations with striking frequency variability. Recently, Stacy *et al* reported the percentage of persons with this null variant was 48 -57%, 23-41%, 32-53% and 40-53% in Caucasians, African Americans, Asians, and Hispanics, respectively [9]. The *GSTM1* homozygous deletion leads to complete absence of enzymatic activity, as demonstrated by the high concordance between GSTM1 phenotype and genotype, particularly with activity toward polycyclic aromatic hydrocarbon oxides/diol epoxide [10-12], compounds which constitute about half of the hydrophobic DNA adducts present in the human urinary bladder (Kadlubar et al., unpublished). Three copies of the *GSTM1* gene are also observed in African Americans and Saudi Arabians, and individuals with this genotype have ultrarapid GSTM1 activities [13, 14].

The incidence of bladder cancer, which varies worldwide, is higher in North America and Europe compared to East Asia [15]. In the United States, bladder cancer is the fourth most common cancer among men [16, 17]. Established risk factors for bladder cancer include cigarette smoking, occupational exposures, and diesel exhaust exposure [18-20]. Clearly, the genes involved in carcinogen metabolism and DNA repair play key roles in the etiology of bladder cancer, a disease induced by exposure to environmental carcinogens.

Based on the role of the GST enzymes in detoxification of carcinogen or environmental pollutants, the copy number variation existing in the *GSTM1* gene may contribute to variable cancer risk resulting from alteration of the expression of GSTM1 protein. To date, numerous molecular epidemiologic studies have focused on the association of susceptibility to cancers, (lung, stomach, urinary bladder, colon, skin, breast, prostate, nasopharynx, oral mucosa, liver, ovary, and leukemias) in relation to the *GSTM1* homozygous deletion, with inconsistent results. However, studies of the exact copy number of *GSTM1* in the genome of individuals who possess the *GSTM1* gene are lacking. In addition to copy number variants, genetic variation in the 5'-promoter region of GSTM1 could influence protein expression levels, but investigation of the role of these genetic variants in development of cancer are limited. We hypothesized that copy number variations combined with other genetic variations in the *GSTM1* promoter contribute to the risk of bladder cancer.

To test this hypothesis, we examined the role of copy number variations combined with promoter SNPs in the *GSTM1* gene in the development of bladder cancer in Caucasians in a large case-control study of bladder cancer.

## Materials and methods

### *Study subjects*

710 Caucasian bladder cancer cases and 782 Caucasian cancer-free controls were identified from an ongoing bladder cancer case-control study (**Table 1**). Bladder cancer patients were recruited from The University of Texas M.D. Anderson Cancer Center and Baylor College of

Medicine. Patient recruitment began in 1999 and is currently ongoing. Cases were newly diagnosed and histologically confirmed urinary bladder cancer patients who had not received prior chemotherapy or radiotherapy before enrollment. There were no recruitment restrictions on age, gender, ethnicity or cancer-stage. The controls have been recruited from the Kelsey-Seybold Clinic, the largest multi-specialty medical organization in Houston, Texas, which provides care to over 400,000 patients at 18 clinic locations. Controls were recruited in a parallel time frame to the cases. Controls were identified by reviewing short survey forms distributed to individuals visiting the clinic for the purpose of health check-ups or for addressing health concerns. On the day of the interview, the controls visited the clinic specifically for the purpose of participating in this study but not for any treatment purposes. Controls had no prior history of cancer (except non-melanoma skin cancer). Smoking status was defined as described in our previous study [21]. Briefly, a never smoker is an individual who never smoked or smoked less than 100 cigarettes in his/her lifetime, a former smoker was one who had quit smoking at least one year before diagnosis with bladder cancer (cases) or interview (controls) and a current smoker was one who was a current smoker or had quit smoking less than one year.

*Determination of GSTM1 copy number*

The gene copy number of *GSTM1* within the genome was determined using a TaqMan copy number assay (Applied Biosystems Inc, Foster City, CA), which used genomic DNA as a template and ran as a duplex TaqMan Real-time PCR reaction with the Rnase P gene as the reference gene, and a well characterized reference sample (Coriell Institute for Medical Research, Camden, NJ) with 2 copies of *GSTM1* as calibrator. Real-time PCR data was analyzed by the comparative Ct method to calculate relative changes in copy number of *GSTM1* [22]. All copy number variant genotypes were determined without knowledge of the case/control status of the subjects.

*Determination of genotypes in the promoter of GSTM1*

The *GSTM1* promoter fragments from each individual were amplified using the forward primer (GSTM1-PF: 5'-CAG GTT GGA CAT TGT TCT CGT G-3') and reverse primer (GSTM1-PR: 5'- CAG CTG CTT CGC ACT TCC CT-3') to produce a 1924 bp fragment and genetic variants were identified by direct sequencing of the PCR products with GenomeLab DTCS Quick Start Kit (Beckman Counter, Fullerton, CA) in a Beckman GeXP Instrument (Beckman Coulter, Fullerton, CA). The sequencing primers were GSTM1-seq1 (5'- GGA GTT TCT TCA GAC TCA CAA T-3'), GSTM1-seq2 (5'-CCT GGG CCT TAA AGC ATG AC-3') and GSTM1-seq3 (5'- CAC AGA CCA CAT TTC CTT TAC-3'). SNPs were identified using CodonCode Aligner software (CodonCode, Dedham, MA), a program for sequence assembly and mutation detection.

*LD block determination and haplotype inference*

The patterns of linkage disequilibrium (LD) between 15 genetic variants in the promoter of *GSTM1* was constructed by HaploView 3.2 using a two marker expectation-maximization algorithm to estimate the maximum-likelihood values in the full-size case-control panel [23]. Haplotypes were inferred by the Phase software based on Bayesian estimation of haplotype frequencies in unphased data [24].

*Statistical methods*

Pearson's $\chi^2$ test for categorical variables and the Student's t test for continuous variables were performed to analyze the differences in distribution between cases and controls. Multivariate logistic regression was used to estimate ORs of copy numbers, genotypes or haplotypes along with 95% CIs, while adjusting for confounding variables such as age, gender, and smoking status. All analyses were performed using the Intercooled Stata 10.0 statistical software package (Stata Co., College Station, TX). All statistical tests were two-sided tests and a *P* value of 0.05 was used as the criterion of statistical significance.

## Results

*Copy number variation and susceptibility to bladder cancer*

*GSTM1* copy numbers were detected using the TaqMan *GSTM1* specific MGB probe. The distribution of *GSTM1* copy number variation in 710

**Table 2.** Copy number frequencies of *GSTM-1* among cases and controls and their association with bladder cancer in Caucasian population.

| Copy Number | Controls (n =782 ) | | Patients (n = 710) | |
|---|---|---|---|---|
| | No. | (%) | No. | (%) |
| 0 | 402 | 51.4 | 381 | 53.7 |
| 1 | 274 | 35.0 | 267 | 37.6 |
| 2 | 79 | 10.1 | 46 | 6.5 |
| $3^+$ | 27 | 3.5 | 16 | 2.2 |
| 1, OR (95% CI)¶ | | | 0.98 (0.78-1.24) | |
| $2^+$, OR (95% CI)¶ | | | 0.56 (0.39-0.81)* | |

¶Data were calculated by unconditional logistic regression and adjusted for sex, age, and smoking status. * *P* value < 0.05.

**Table 3.** List of genetic variants in the promoter of GSTM1 identified in Caucasians.

| Genetic Variants | | Position * | Rs No. | MAF (%) |
|---|---|---|---|---|
| 1 | INS | -1542 | NA | 40.0 |
| 2 | C/G | -1529 | rs36210087 | 7.3 |
| 3 | A/G | -1490 | rs36209763 | 19.3 |
| 4 | A/G | -1143 | rs36209754 | 24.5 |
| 5 | A/T | -888 | NA | 11.0 |
| 6 | C/G | -498 | rs412543 | 14.6 |
| 7 | C/G | -486 | rs3815029 | 9.9 |
| 8 | C/T | -471 | NA | 12.8 |
| 9 | A/G | -426 | rs412302 | 26.7 |
| 10 | C/T | -344 | rs4147561 | 28.5 |
| 11 | A/T | -343 | rs4147562 | 25.8 |
| 12 | C/T | -341 | NA | 15.9 |
| 13 | C/T | -339 | rs4147563 | 47.3 |
| 14 | G/A | -304 | rs28529287 | 18.5 |
| 15 | T/C | -164 | rs36208869 | 22.5 |

* Upstream of the ATG start site of GSTM1 gene.

cases and 782 controls are shown in **Table 2**. The frequencies of subjects with 0, 1 and at least 2 copies of *GSTM1* were 51.4%, 35.0%, 13.6%, respectively in controls, and 53.7%, 37.6%, 8.7%, respectively in cases. There was no significant difference in the frequency of 1-copy carriers of *GSTM1* between controls and cases. However, subjects with at least 2 copies of *GSTM1* were more prevalent in controls than in patients (13.6% versus 8.7%, $P < 0.01$). Furthermore, unconditional logistic regression demonstrated that the subjects with at least 2 copies of the gene had significantly decreased risk of bladder cancer compared to those with the *GSTM1* null genotype (OR, 0.56, 95% CI, 0.39-0.81) (**Table 2**). This suggests a possible dominant effect of this copy number variant on the development of bladder cancer.

*GSTM1 promoter variants and haplotype structure*

Direct sequencing of the *GSTM1* promoter revealed 15 genetic variants (**Table 3**), eleven of which are deposited in NCBI database while four are novel. The minor-allele frequencies of those SNPs are between 7% and 50%. As depicted by Haploview software, four LD blocks are identified across the 2-kb region in the promoter of *GSTM1* (**Figure 1**). The strongest LD block (Block 4) with highest D' value is located in the proximal promoter of the *GSTM1* gene.

*Association of bladder cancer risk with individual genotypes and haplotypes*

Employing CNVs and LD block analyses of the

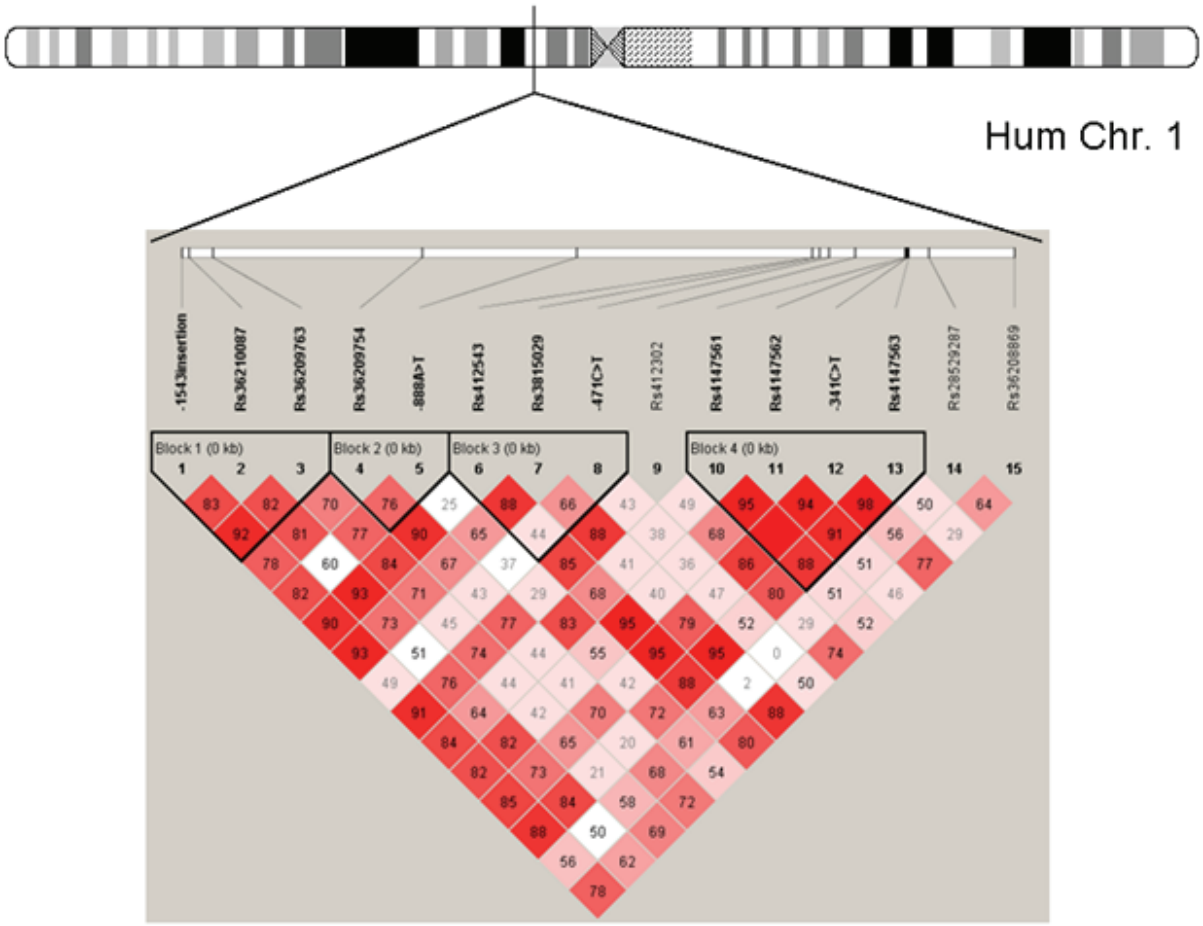


**Figure 1.** Diagram of LD block structure of *GSTM-1* promoter in chromosome 1p13.3. LD blocks were identified by strong LD using Haploview software. Depth of red color, computed pairwise D' value; deeper red, higher D'.

*GSTM1* promoter, we examined the association between individual genotypes and haplotypes and bladder cancer risk. There was no significant association between any individual allele and bladder cancer risk (data not shown). We then examined the possibility that the distribution of haplotypes of the LD blocks differed between cases and controls. As with the SNP data, no single haplotype of the *GSTM1* promoter was associated with bladder cancer risk (data not shown).

*Association of bladder cancer risk with individual alleles and haplotypes stratified by copy number variation*

We next analyzed the joint effect of each promoter variant and copy number variant of the *GSTM1* gene. No SNP in the *GSTM1* promoter was associated with decreased bladder cancer risk in individuals carrying one copy of *GSTM1*. However, subjects with two copies, combined with the -1542 insertion genotype had significantly decreased risk for development of bladder cancer (OR, 0.58; 95%CI, 0.32-0.10), and similar results were found in two copy carriers with -888 A, -486G, - 344 C, -343 A, -341 C alleles (**Table 4**).

To further explore the relationship between the *GSTM1* promoter SNPs combined with CNV and the risk of bladder cancer, we tested the distribution differences of estimated *GSTM1* promoter haplotype frequencies stratified by CNV between bladder cancer patients and normal controls (**Table 5**). In LD block one, the genotype with the $INS_{-1543}$-$C_{-1529}$-$A_{-1429}$ haplotype combined with two copies of the gene is associated with significantly reduced risk of bladder cancer (OR, 0.45; 95%CI, 0.21-0.94). Similarly, in the

**Table 4.** *GSTM1* promoter SNPs and the risk of bladder cancer in two-copy *GSTM1* group

| Genetic Variations | Controls (n =156 ) | Bladder Cancer (n =92 ) | | |
|---|---|---|---|---|
| | No. (%) of allele | No.(%) of allele | OR† (95% CI) | *P* value |
| -1543 INS | | | | |
| 0 INS | 93 (59.6) | 66 (71.7) | Ref. | |
| 1 INS | 63 (40.4) | 26 (28.3) | 0.58 (0.32-1.00) | 0.05 |
| -888 A>T | | | | |
| A | 141 (90.4) | 73 (79.3) | Ref. | |
| T | 15 (9.6) | 19 (20.7) | 2.45 (1.11-5.42) | 0.01 |
| -486 C>G | | | | |
| C | 136 (87.2) | 87 (94.6) | Ref. | 0.05 |
| G | 20 (12.8) | 5 (5.4) | 0.37 (0.14-1.01) | |
| -344 C>T | | | | |
| C | 111 (71.1) | 47 (51.1) | Ref. | 0.001 |
| T | 45 (28.9) | 45 (48.9) | 2.36 (1.34-4.18) | |
| -343 A>T | | | | |
| A | 121 (77.6) | 55 ( 59.8) | Ref. | 0.003 |
| T | 35 (22.4) | 37 (40.2) | 2.33 (1.28-4.24) | |
| -341 C>T | | | | |
| C | 130 (83.3) | 65 (70.7) | Ref. | 0.019 |
| T | 26 (16.7) | 27 (29.3) | 2.08 (1.07-4.02) | |

† Data were calculated by logistic regression

group with two copies, significantly increased risk of bladder cancer was observed in the subjects with haplotype $A_{-1143}$-$T_{-888}$ (OR, 2.89; 95% CI, 1.24-6.81) in LD block 2; decreased risk with haplotype $C_{-498}$-$C_{-486}$-$T_{-471}$ (OR, 0.17; 95% CI, 0.03-0.72) in LD block 3; decreased risk with haplotype $C_{-344}$-$A_{-343}$-$C_{-341}$-$C_{-339}$ (OR, 0.31; 95%CI, 0.12-0.79) and decreased risk with haplotype $C_{-344}$-$A_{-343}$-$C_{-341}$-$T_{-339}$ (OR, 0.40; 95%CI, 0.19-0.86) in LD block 4. These data clearly suggest a joint effect of copy number variation and promoter SNPs on the risk of bladder cancer.

## Discussion

In the present study, no statistically significant association was found when we analyzed the relationship between any single SNP or haplotype and risk of bladder cancer. However, when stratified by copy number, we found that the -1543 insertion variant, -888 A, -486G, - 344 C, -343 A, -341 C alleles were related to decreased risk of bladder cancer among those with two copies of *GSTM1*, but not among those with one copy. This implies that not only copy number variants, but also several promoter genetic variants in *GSTM1* contribute to bladder cancer risk. However, the functional basis of this association is unclear, and is the subject of future investigations in our laboratory.

The earliest published studies of *GSTM1* and bladder cancer risk were focused on the influence of the homozygous deletion (*GSTM1 null* genotype) of *GSTM1* on risk. Two recently published meta-analyses demonstrated that *GSTM1 null* status was associated with a modest increase in the risk of bladder cancer; however, not all studies had positive results, with the reported ORs falling between 0.76 and 5.00 [25, 26]. The current study assessed the association between CNVs of *GSTM1*, not just the presence/absence of *GSTM1*, and the risk of bladder cancer. Our data demonstrated that subjects with two or more copies of *GSTM1* had significantly decreased bladder cancer risk, compared to both the homozygous deletion of the gene and to one copy gene carriers. This finding is supported by McLellan and colleagues' functional study that demonstrated that individuals with at least two copies of *GSTM1* exhibited elevated enzyme activity compared with one copy *GSTM1* carriers [13]. They collected 15 samples from Saudi Arabian subjects and found the subjects with at least two copies of *GSTM1* have 1.7 fold higher GSTM1 activities than those who carry one copy of *GSTM1*. To further verify the influence of

**Table 5.** *GSTM1* promoter haplotype and the risk of bladder cancer in two-copy *GSTM1* group

| Haplotype | Controls (n =156 ) | Bladder Cancer (n = 92) | | |
|---|---|---|---|---|
| | No. (%) | No.(%) | OR† (95% CI) | *P* value |
| Haploblock1 | | | | |
| 0 $INS_{-1543}$-$C_{-1529}$-$A_{-1490}$ | 59 (37.8) | 39 (42.4) | Ref. | |
| 0 $INS_{-1543}$_$C_{-1529}$-$G_{-1490}$ | 34 (21.8) | 24 (26.1) | 1.07 (0.52-2.18) | 0.84 |
| 1 $INS_{-1543}$_$C_{-1529}$-$A_{-1490}$ | 54 (34.6) | 16 (17.4) | 0.45 (0.21-0.94) | 0.02 |
| others | 9 (5.8) | 13 (14.1) | N/A | N/A |
| Haploblock2 | | | | |
| $A_{-1143}$-$A_{-888}$ | 99 (63.5) | 44 (47.8) | Ref. | |
| $A_{-1143}$-$T_{-888}$ | 14 (9.0) | 18 (19.6) | 2.89 (1.24-6.81) | 0.006 |
| $G_{-1143}$-$A_{-888}$ | 42 (26.9) | 29 (31.5) | 1.55 (0.82-2.93) | 0.14 |
| $G_{-1143}$-$T_{-888}$ | 1 (0.6) | 1 (1.1) | N/A | N/A |
| Haploblock 3 | | | | |
| $C_{-498}$-$C_{-486}$-$C_{-471}$ | 103 (66.0) | 64 (69.6) | Ref. | |
| $C_{-498}$-$C_{-486}$-$T_{-471}$ | 13 (8.3) | 8 (8.7) | 0.99 (0.35-2.73) | 0.98 |
| $C_{-498}$-$G_{-486}$-$T_{-471}$ | 19 (12.2) | 2 (2.2) | 0.17 (0.03-0.72) | 0.009 |
| $G_{-498}$-$C_{-486}$-$C_{-471}$ | 20 (12.8) | 13 (14.1) | 1.05 (0.45-2.33) | 0.91 |
| others | 1 (0.7) | 5 (5.4) | N/A | N/A |
| Haploblock 4 | | | | |
| $T_{-344}$-$T_{-343}$-$T_{-341}$-$C_{-339}$ | 24 (15.4) | 26 (28.3) | Ref. | |
| $C_{-344}$-$A_{-343}$-$C_{-341}$-$C_{-339}$ | 36 (23.0) | 12 (13.0) | 0.31 (0.12-0.79) | 0.006 |
| $C_{-344}$-$A_{-343}$-$C_{-341}$-$T_{-339}$ | 73 (46.8) | 32 (34.8) | 0.40 (0.19-0.86) | 0.010 |
| $T_{-344}$-$A_{-343}$-$C_{-341}$-$C_{-339}$ | 9 (5.8) | 7 (7.6) | 0.72 (0.20-2.55) | 0.57 |
| $T_{-344}$-$T_{-343}$-$C_{-341}$-$C_{-339}$ | 9 (5.8) | 8 (8.7) | 0.82 (0.24-2.82) | 0.73 |
| others | 5 (3.2) | 7 (7.6) | N/A | N/A |

† Data were calculated by logistic regression.

*GSTM1* copy number to GSTM1 expression, we conducted expression analysis using HPLC and found that individuals with at least two copies of *GSTM1* have 2-fold higher GSTM1 expression than those with one copy. Although these studies provide support for a gene-dose effect, the wide variability seen in GSTM1 expression within *GSTM1* copy number categories also lend support to the idea of other genetic variants contributing to GSTM1 protein expression.

Gene-environment interaction plays an important role in the etiology of bladder cancer. Many epidemiologic studies have shown that bladder cancer is related to environmental factors such as cigarette smoking and dietary habits, especially the consumption of isothiocyanates from cruciferous vegetables [27-30]. GSTM1 is involved in the phase II detoxification of many environmental carcinogens including polycyclic aromatic hydrocarbons in cigarette smoke, so the lack of GSTM1 activity may decrease the detoxification of tobacco carcinogens and consequently elevate the risk of bladder cancer among smokers. Previous studies provided evidence of an interaction between *GSTM1 null* genotype and ever smoking in relation to elevated risks, especially in heavy smokers [31, 32]. Aside from smoking status, yellow-orange vegetable and fruit intake also reduce the risk of bladder cancer and this association was stronger among individuals with the GSTM1 present than the null genotype [29].Thus, the genetic contribution to *GSTM1* variation could be confounded by environmental factors such as smoking status and dietary habits.

In our liver GSTM1 expression analysis, there was a 145-fold variation in protein expression (data not shown). Copy number variation and environmental factors may partially explain this variation, but cannot account for the total variation. Among one copy number carriers, there still was an 80-fold variation in GSTM1 expression. Thus, other genetic factors could similarly contribute to this expression variability. Generally, functional promoter genetic variants could alter the binding ability of transcriptional factors to the gene promoter and subsequently influence gene expression. In 2003, Bartley and colleagues analyzed the promoter of *GSTM1* and experimentally indicated that *GSTM1* could be activated by Myb oncoprotein [33]. Our sequencing results showed that 1 insertion variant and 14 SNPs exist in the promoter of the *GSTM1* gene. In the region -300bp to -500bp,

there were 9 SNPs which may affect the transcriptional factor binding. Among these 15 genetic variants, -498 C>G SNP has been experimentally demonstrated to alter a AP2 nuclear protein binding site and significantly influence *GSTM1* transcriptional activity [34]. In addition, the -304 A>G SNP could alter a HNF-4 binding site and might change the *GSTM1* transcriptional activity. Furthermore, the functions of these genetic variants might vary by different transcriptional factors in different tissues or under varying physiological conditions. Thus, functional studies to biochemically characterize these genetic variants are essential.

Other laboratories have reported that the -498 C allele creates a AP2 binding site and subsequently enhances the promoter activity about 2-3 fold *in vitro* and *in vivo* [34]. This is consistent with our present case-control study, in which we found that Caucasian carriers of the -498 C allele with two copies of *GSTM1* had significantly decreased bladder cancer risk (OR, 0.56; 95% CI, 0.39-0.81). In contrast, the former study found that the -498 C allele was associated with increased breast cancer risk in Chinese. These inconsistent results may be due to population-specific differences that result from different gene-gene interactions, or may be due to different gene-environment interactions. It is also possible that other functional promoter variants exist in the Chinese population that are extremely rare or absent in Caucasians.

In summary, this report examines the effect of promoter genetic variants combined with copy number variation in *GSTM1* on the risk of bladder cancer. Regulation of *GSTM1* expression and its influence on risk of bladder cancer are complicated by the various genetic variants, not only copy number variation but also SNP/or insertion, and environmental factors in different ethnic populations. Our study has limitations due to its limited sample size, so the possible combined effects of copy number variation and promoter genetic polymorphisms of *GSTM1,* and/or environmental exposures on risks of bladder cancer should be further confirmed by larger studies.

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