RESEARCH ARTICLE

CYP1A1 (Ile^{462}Val), CYP1B1 (Ala^{119}Ser and Val^{432}Leu), GSTM1 (null), and GSTT1 (null) Polymorphisms and Bladder Cancer Risk in a Turkish Population

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Abstract

We aimed to investigate bladder cancer risk with reference to polymorphic variants of cytochrome p450 (CYP) 1A1, CYP1B1, glutathione S-transferase (GST) M1, and GSTT1 genes in a case control study. Polymorphisms were examined in 114 bladder cancer patients and 114 age and sex-matched cancer-free subjects. Genotypes were determined using allele specific PCR for CYP1A1 and CYP1B1 genes, and by multiplex PCR and melting curve analysis for GSTM1 and GSTT1 genes. Our results revealed a statistically significant increased bladder cancer risk for GSTT1 null genotype carriers with an odds ratio of 3.06 (95% confidence interval=1.39-6.74, p=0.006). Differences of CYP1A1, CYP1B1 and GSTM1 genotype frequencies were not statistically significant between patients and controls. However, the specific combination of GSTM1 null, GSTT1 null, and CYP1B1 codon 119 risk allele carriers and specific combination of GSTM1 present, GSTT1 null, and CYP1B1 432 risk allele carriers exhibited increased cancer risk in the combined analysis. We did not observe any association between different genotype groups and prognostic tumor characteristics of bladder cancer. Our results indicate that inherited absence of GSTT1 gene may be associated with bladder cancer susceptibility, and specific combinations of GSTM1, GSTT1 and CYP1B1 gene polymorphisms may modify bladder cancer risk in the Turkish population, without any association being observed for CYP1A1 gene polymorphism and bladder cancer risk.

Keywords: Bladder cancer - CYP1A1 - CYP1B1 - GSTM1 - GSTT1

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Introduction

Cytochrome p450 (CYP), and glutathione S-transferase (GST) gene superfamilies encode metabolic enzymes which play a major role in cellular detoxification mechanism. They catalyze reactions with common carcinogens that have been claimed to involve in bladder cancer (BC) etiology such as polycyclic aromatic hydrocarbons and aminobiphenyls (Boffetta et al., 1997; Feng et al., 2002). Single nucleotide polymorphisms and inherited loss of both alleles are common in these gene superfamilies with varying frequencies among different populations (Senthilkumar and Thirumurugan, 2012; Sharma et al., 2012; Zhou et al., 2012). These genetic alterations lead to a change or complete loss in activity of the corresponding enzymes and result in impaired cellular detoxification which give rise to a loss in cancer prevention mechanisms. Chemical carcinogens are mostly activated to electrophilic reactive forms that can cause DNA damage by phase I enzymes. These intermediate products are subsequently deactivated by phase II enzymes (Turesky and Le Marchand, 2011). Since activities of phase I and phase II enzymes are affected by genetic variations, the polymorphic enzyme variants in metabolic pathways are supposed to be responsible for the difference in cancer development risk between different individuals. CYP1A1 and CYP1B1 enzymes belong to cytochrome p450 enzyme family, and they are responsible for the activation of procarcinogens to reactive metabolites (Turesky and Le Marchand, 2011). CYP1A1 gene Ile^{462}Val polymorphism and CYP1B1 gene Ala^{119}Ser and Val^{432}Leu variants are linked to increased risk for developing lung, bladder, and head and neck cancers (Bartsch et al., 2000; Ji et al., 2012; Salinas-Sanchez et al., 2012; Shukla et al., 2012). GSTM1 and GSTT1 genes are also implied to be predisposing risk factors for several cancer types due to the inherited loss of both alleles (null genotype) that result in complete lack of enzymatic activity. However, the data about these metabolic gene polymorphisms and their relevance to BC development is limited in Turkish population. Therefore, we conducted this study to investigate the association between CYP1A1, CYP1B1, GSTM1 and GSTT1 gene polymorphisms and bladder cancer risk in a Turkish population.

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Materials and Methods

Patients and controls

With the approval from Institutional Ethics Comittee (Reference number: 2012-104), we selected 114 cancer-free control individuals (103 male, 11 female) and 114 histologically diagnosed BC patients (103 male, 11 female) for this study which was performed in accordance with Declaration of Helsinki and informed consents were obtained from the participants appropriately. Patients with any cancer at other body sides were excluded from the study. Clinical and demographic data including gender, age, smoking status, pathologic tumor stage and tumor grades were obtained for statistical analysis. All of the patients and control subjects were of Turkish origin.

DNA isolation and genotyping assays

Genomic DNA was isolated from formallin fixed paraffin embedded (FFPE) nontumoral bladder tissues for BC patients by using QIAamp DNA FFPE tissue kit (Qiagen); and from blood buffy coat for control group by using QIAamp Mini kit (Qiagen), according to the manufacturer's instructions.

Ile^{462}Val polymorphism (rs1048943) variants in CYP1A1 gene (Unigene ID: Hs.72912) were determined by allele specific PCR (ASPCR) as previously described (Hirvonen et al., 1992). Two primers; 5'-AAGACCTCCCAGCGGGCAAT-3' and 5'-AAGACCTCCCAGCGGGCAAC-3' with different bases at 3' end corresponding to A to G transition in exon 7 were enrolled with a common reverse primer (5'-GAAAGGCTGGGTCCACCCTCT-3'). PCR was performed at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 sec, 65 °C for 45 sec, 72 °C for 30 sec and a final extension at 72 °C for 4 min.

CYP1B1 (UniGene ID Hs.154654) polymorphism variants in codon 119 (rs1056827) were determined by ASPCR as previously described (Tanaka et al., 2002). Primers; 5'-GGCCTTCGCCGACCGGCCGG-3' and 5'-GGCCTTCGCCGACCGGCCGT-3' with different bases at 3' end corresponding to G to T transition were enrolled with a common reverse primer (5'-GAAGTTGCGCATCATGCTGT-3'). PCR was performed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 69 °C for 30 sec, 72 °C for 30 sec and a final extension at 72 °C for 4 min.

CYP1B1 gene Leu432Val polymorphism (rs1056836) was determined by ASPCR method as described (Tanaka et al., 2002). Primers; 5'-TCCGGGTTAGGCCACTTCAG-3' and 5'- TCCGGGTTAGGCCACTTCAC-3' with different bases at 3' end were used to detect C to G transition with a common reverse primer (5'-TATGGAGCACACCTCACCTG-3'). PCR was performed at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 30 sec and a final extension at 72 °C for 4 min.

After amplification of CYP1A1 and CYP1B1 genes, all PCR products were subjected to electrophoresis on 2% agarose gel to visualize the generated bands that correspond to prevalent or rare alleles.

Deletions in both alleles of GSTM1 (UniGene ID Hs.301961) and GSTT1 (UniGene ID Hs.268573) genes were screened by multiplex PCR and melting curve analysis method as previously described (Marin et al., 2010). Primers for GSTM1 gene were 5'-GAACTCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGCTCAAATATACGGTGG-3'. For amplification of GSTT1 gene, 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'- GGAAAAGGGTACAGACTGGGGA-3' primers were used. The primer pairs were mixed in a single tube. With this approach both amplicons served as an internal control in each tube for PCR efficiency. In cases with no amplification was observed (ie. null - null genotype), seperate PCR amplifications were performed for both genes using bcl2 as an internal control gene to evaluate the DNA integrity. Amplification protocol was consisted of an activation step at 94 °C for 5 minutes and 40 cycles with amplification steps at 94 °C, 62 °C, and 72 °C for 30 sec each. Amplifications were carried out on RotorGene Q 5-Plex (Qiagen) in a final volume of 25 μL containing 40-100 ng of genomic DNA. Amplified products were determined by visual analysis of melting curves generated between 65°C to 95°C at a ramping rate of 0.1°C/sec.

Statistical analysis

The genotype distributions of CYP1A1 and CYP1B1 genes among control subjects were analysed by chi-square test to determine whether they were in Hardy-Weinberg equilibrium. Continious variables were compared by "t test" between patient and control groups. The estimates of associations between each genotype and susceptibility to BC risk were obtained from unconditional logistic regression models using odds ratios (ORs) adjusted for sex, smoking habit, and age with a confidence interval (CI) of 95%. Cancer risk for specific genotype combinations of GSTM1 and GSTT1 genes were assessed by stratifying genotypes into four groups, as wild for both (+/+) (reference category), wild/null (+/-), null/wild (-/+), and null for both (-/-). The effects of CYP1A1 and CYP1B1 genotypes on BC risk for combined GST genotype groups were also analysed by stratifying cancer patients and controls to have a risk allele for each CYP gene or not. Pathological tumor stages were dichotomised as non-invasive (stage pTa) vs. invasive (stages pT1-pT4) for statistical analysis. The associations of different genotypes with histological tumor grades and tumor stages were tested using the chi-square and Fisher exact tests. Statistical analyses were performed using "SPSS for Windows software version 11.5", and $p<0.05$ was considered significant.

Results

CYP1A1 Ile^{462}Val, CYP1B1 Ala^{119}Ser and Val^{432}Leu, GSTM1 null and GSTT1 null genotypes were analysed in 114 BC patients and 114 control subjects. Baseline characteristics of the study population are summarized in Table 1. The mean age was 65.1±9.25 for BC patients, and 63.2±5.75 for control group, and the difference was not statistically significant ($p>0.05$). There were more smokers in the BC patient group (n=97) than in control group (n=54) ($p<0.01$). For histological grading, 62 (54.4%)

patients had low grade, and 52 (45.6%) patients had high grade urothelial carcinomas. As for pathological tumor staging, 69 (60.5%) patients had stage pTa, 32 (28.1%) had stage pT1, and 13 (11.4%) had stage ≥pT2 tumors.

Genotype frequencies in case and control groups and their relation to BC risk are shown in Table 2. Among controls, the genotype distributions of CYP1A1 and CYP1B1 polymorphisms were in Hardy-Weinberg equilibrium. Distributions of CYP1A1, CYP1B1 codon 119 and GSTM1 genotypes were similar in case and control groups. For CYP1B1 codon 432 polymorphism, risk allele was slightly more common in patient group with an OR of 1.40 (95%CI: 0.77-2.57) (p=0.22). The frequency of GSTT1 null genotype was significantly higher in BC cases (27.2%) than in controls (14.0%) with an OR of 3.08 (95%CI: 1.40-6.78) (p=0.005). There were no statistically significant differences between different genotype groups of BC patients with regard to tumor grades or tumor stages (all p>0.05).

As presented in Table 3, the results of combined GSTM1 and GSTT1 genotype analysis revealed that GSTT1 null genotype was significantly associated with increased BC risk, irrespective of the GSTM1 genotype status. In the analysis for combined effects of CYP polymorphisms on GSTM1/GSTT1 genotypes, two specific combinations were significantly associated with elevated risk for BC (Table 3). The highest OR was observed for GSTM1 null, GSTT1 null, and CYP1B1 codon 119 risk allele carriers (OR=18.9, 95%CI: 1.65-217.9) (p=0.02). In addition, the OR for BC risk was 9.07 (95%CI: 1.97-41.9) in GSTM1 present, GSTT1 null, and CYP1B1 codon 432 risk allele carriers (p<0.01). CYP1A1 polymorphic variants did not exhibit an elevated BC risk in the combined analysis.

Table 1. Baseline Characteristics of the Study Population

Characteristic		Controls (n=114) no (%)	BC patients (n=114) no (%)	p value
Age (years)				0.055
Mean±sd		63.2±5.75	65.1±9.25	
Range		49-80	44-87	
Gender	Male	103 (90.4)	103 (90.4)	1.00
	Female	11 (9.6)	11 (9.6)	
Smoking habit	Ever	54 (47.4)	97 (85.1)	0.00
	Never	60 (52.6)	17 (14.9)	
Tumor grade	Low		62 (54.4)	
	High		52 (45.6)	
Tumor stage	Non-invasive (pTa)		69 (60.5)	
	Invasive (pT1-pT4)		45 (39.5)	

Table 2. The Frequency Distributions of Studied Gene Polymorphisms and Their Odds Ratios and 95%CIs for BC Risk in Case and Control Groups

Genotype		Controls n (%)	BC patients n (%)	OR*	95%CI	p value
CYP1A1	AA	95 (83.3)	92 (80.7)		(reference)	
	AG	18 (15.8)	20 (17.5)	1.21	0.52-2.78	0.66
	GG	1 (0.88)	2 (1.75)	1.30	0.11-15.9	0.84
	AG+GG	19 (16.7)	22 (19.3)	1.17	0.53-2.58	0.69
CYP1B1 (119G/T)						
	GG	60 (52.6)	58 (50.9)		(reference)	
	GT	44 (38.6)	45 (39.5)	1.11	0.58-2.12	0.76
	TT	10 (8.77)	11 (9.65)	1.13	0.43-3.55	0.70
	GT+TT	54 (47.4)	56 (49.1)	1.10	0.60-2.00	0.77
CYP1B1 (432C/G)						
	CC	65 (57.0)	58 (48.9)		(reference)	
	CG	37 (32.5)	41 (36.0)	1.11	0.41-2.99	0.84
	GG	12 (10.5)	15 (13.1)	1.53	0.78-2.99	0.22
	CG+GG	49 (43.0)	56 (49.1)	1.40	0.77-2.57	0.27
GSTM1	Present	63 (55.3)	60 (52.6)		(reference)	
	Null	51 (44.7)	54 (47.4)	1.20	0.66-2.21	0.55
GSTT1	Present	98 (86.0)	83 (72.8)		(reference)	
	Null	16 (14.0)	31 (27.2)	3.08	1.40-6.78	0.005

*Odds ratio adjusted for age and smoking status. n: Number; BC: Bladder cancer; OR: Odds ratio; CI: Confidence interval

Table 3. Odds Ratios and 95%CIs in BC Patients and Controls for Combined Genotypes of GSTM1 and GSTT1 Genes and Significant Associations with CYP Genes

Genotype	Controls n (%)	BC patients n (%)	OR*	95%CI	p value
GSTM1/GSTT1					
+/+	54 (47.4)	40 (35.1)	1.00	(reference)	
-/+	44 (38.6)	43 (37.7)	1.27	0.65-2.46	0.48
+/-	9 (7.89)	20 (17.5)	3.64	1.32-10.0	0.01
-/-	7 (6.14)	11 (9.64)	3.23	0.99-10.5	0.051
GSTM1/GSTT1/CYP1B1 (119G/T)					
+/+/GG	25 (21.9)	17 (14.9)	1.00	(reference)	
-/-/GT+TT	1 (0.88)	5 (4.39)	18.9	1.65-217.9	0.02
GSTM1/GSTT1/CYP1B1 (432C/G)					
+/+/CC	31 (27.2)	23 (20.2)	1.00	(reference)	
-/-/CG+GG	3 (2.63)	15 (13.2)	9.07	1.97-41.9	0.005

*Odds ratio adjusted for age and smoking status. n: Number; BC: Bladder cancer; OR: Odds ratio; CI: Confidence interval

Discussion

Urothelial tumorigenesis is a complex, multistep and multifactorial event, in which different somatic mutations, toxic carcinogenic chemicals, and inflammatory agents are implied to play a role in its ethiogenesis (Lopez-Beltran et al., 2008; Botelho et al., 2010; Pollard et al., 2010). Cigarette smoking is one of the major environmental risk factors for BC due to the presence of procarcinogens in tobacco. However, besides the strong association between cigarette smoking and urothelial carcinogenesis, BC do not develop in majority of the smokers. This finding suggests a crucial role for carcinogen metabolism and DNA repair pathways to modify the BC risk among different individuals.

The cascade of procarcinogen metabolism starting with activation of xenobiotics to genotoxic intermediate products, followed by conversion to less toxic diols and finally to nontoxic compounds is a complex process. While the activating polymorphisms in phase I enzymes are resulted in accumulation of intracellular genotoxic metabolites, conversely a decrease or loss in ezymatic activity of phase II enzymes by polymorphic variants lead to a less effective removal of these activated toxic metabolites. Ile^{462}Val polymorphism occurs near the catalytic region of the CYP1A1 enzyme, and results in a 2-fold elevated enzymatic activity in variant genotype (Bartsch et al., 2000). Ala^{119}Ser, and Val^{432}Leu polymorphisms in CYP1B1 gene also lead to the formation of variant enzymes with increased enzymatic activities (Hanna et al., 2000). As presumed, variant genotypes

of these phase I enzymes were found to be associated with increased risk for several cancer types (Bartsch et al., 2000). In contrast to phase I enzymes, predicting the net effect of GSTM1 and GSTT1 enzymes in cancer susceptibility is difficult due to their multifunctional roles in metabolic pathways. Although, they are primarily involved in detoxification of intermediate products, they also participate in the activation of procarcinogens to genotoxic metabolites such as halogenated alkanes (Rebbeck et al., 2009).

Since activation and detoxification of carcinogens are mainly influenced by the activity of phase I and phase II enzymes, we aimed to investigate whether polymorphisms of CYP1A1, CYP1B1, GSTM1 and GSTT1 genes modify the BC risk in a Turkish population. All selected genes encode enzymes which are participated in metabolism of xenobiotics known to be related with urothelial carcinogenesis as candidates for cancer susceptibility.

CYP1A1 Ile^{462}Val polymorphic variants are mostly linked to tobacco related cancers such as lung and esophageal cancer in different ethnic populations (Yang et al., 2005; Dong et al., 2008; Lee et al., 2008). In contrast, the results of previous studies among Turkish population demonstrated increased risks for developing ovarian and prostatic carcinomas (Aktas et al., 2002; 2004) but no association was detected with lung cancer (Atinkaya et al., 2012). The results of our study also did not reveal an increased risk of BC for CYP1A1 Ile^{462}Val variant genotype. In addition, CYP1A1 gene transcription is proposed to be strongly induced by 2,4,7,8-tetrachlorodibenzo-para-dioxin in GSTM1 null genotype carriers which may lead to an additive risk for cancer development in CYP1A1 variant genotype. However, combined genotype analysis also did not demonstrate an increased risk for GSTM1 null and CYP1A1 variant allele carriers.

We found no difference in frequencies of CYP1B1 Ile119Val genotypes between BC patients and controls. Studies on Val^{432}Leu polymorphism of CYP1B1 gene reported inconsistent results with respect to the BC risk. Our data, however, suggest that Val^{432}Leu polymorphism in CYP1B1 gene does not associate with an increased risk for BC development. We did not observe an increased BC risk between null and wild types of GSTM1 gene. In previous studies, while some investigators reported an association between null genotype and overall BC risk (Toruner et al., 2001; Cengiz et al., 2007), another did not (Altayli et al., 2009). GSTT1 null genotype was reported to be associated with increased BC risk among smokers in Turkish population (Altayli et al., 2009). Our results strongly support the predisposing role of GSTT1 null genotype for BC development in Turkish population, too.

We also searched for the combined effects of selected polymorphisms for cancer susceptibility and their association between tumor grades and tumor stages in BC. The combination of the two high-risk GST genotypes (GSTM1/GSTT1 double null) increased BC risk with an OR of 3.23. However, OR for BC risk was 3.64 for patients with GSTM1 present/GSTT1 null genotype. These findings suggest that BC risk was mainly influenced by GSTT1 null genotype, irrespective of the GSTM1 status. The data of the present study revealed potantial increased cancer risks for two additional specific genotype combinations: GSTM1 null, GSTT1 null, and CYP1B1 codon 119 risk allele carriers exhibited the highest risk with an OR of 18.9. Secondly, the OR for BC risk was 9.07 (95%CI: 1.97-41.9) in GSTM1 present, GSTT1 null, and CYP1B1 codon 432 risk allele carriers.

In a previous study, GSTM1 null and GSTM1/GSTT1 double null genotypes were reported to be significantly associated with tumor grade of BC in Chinese population (Song et al., 2009). In that study also a significant association was found between NAT2 slow-acetylator genotype and higher tumor stage in BC patients (Song et al., 2009). However, we found that none of the studied genotypes were associated with tumor grade or tumor stage in Turkish BC patients.

In conclusion, our results indicate that inherited absence of both alleles in GSTT1 gene, and specific combinations of GSTM1, GSTT1 and CYP1B1 gene polymorphisms may increase bladder cancer risk in Turkish population, but no association was observed with CYP1A1 gene polymorphism, either alone or in combined genotype analysis.

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