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Common Polymorphisms in *GSTA1*, *GSTM1* and *GSTT1* Are Associated with Susceptibility to Urinary Bladder Cancer in Individuals from Balkan Endemic Nephropathy Areas of Serbia

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Balkan endemic nephropathy (BEN) is a chronic familial form of interstitial nephritis that might eventually lead to end stage renal disease. This nephropathy affects individuals living along of the Danube River and its tributaries in Serbia, Bosnia, Croatia, Bulgaria and Romania. The increased incidence of urinary tract tumors in the BEN areas is well described, but its specific genetic predisposition is still unclear. Certain nephrocarcinogenic compounds, including those associated with BEN, are metabolized by glutathione S-transferase (GST) superfamily of phase II detoxication enzymes. Importantly, the GST-mediated detoxification may result in formation of more toxic compounds. We examined the association of common *GST* polymorphisms and bladder cancer (BC) risk in individuals from BEN areas in Serbia. A hospital-based case-control study included 201 BC cases (67 from BEN region) and 122 controls. Each polymorphism was identified by a PCR-based method. Individuals from BEN region with low-expression *GSTA1* genotype (*AB+BB*) exhibited a 2.6-fold higher BC risk compared to those with *GSTA1* (*AA*) genotype who were from non-BEN region (OR = 2.60, p = 0.015). In contrast, carriers of *GSTM1-active* genotype from BEN region had a 2.9-fold increased BC risk compared to those with *GSTM1-active* genotype from non-BEN region (OR = 2.90, p = 0.010). Likewise, carriers with *GSTT1-active* genotype from BEN region exhibited 2.1-fold higher BC risk compared to those from non-BEN region with *GSTT1-active* genotype (OR = 2.10, p = 0.027). Thus, common polymorphisms in *GSTA1*, *GSTM1* and *GSTT1* are associated with susceptibility to BC in individuals from BEN areas of Serbia.

Keywords: Balkan endemic nephropathy; glutathione transferase; polymorphism; risk; urinary bladder cancer
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Introduction

Balkan endemic nephropathy (BEN) is a chronic familial form of interstitial nephritis that might eventually lead to end stage renal disease (Stefanovic and Cosyns 2004). This nephropathy represents an endemic disease affecting individuals living along of the Danube River and its tributaries in Serbia, Bosnia, Croatia, Bulgaria and Romania (Jelakovic et al. 2014). Ever since the 1950s, when the disease was first described, its geographic distribution has remained the same. The most important epidemiologic characteristics of BEN are its focal appearance in certain villages and small towns in endemic region, in residents who have lived there for at least 15 years, as well as, familial character of the disease, although it is not inheritable (Stefanovic and Cosyns 2004). Moreover, BEN is associated with increased incidence of urinary tract transitional cell carcinoma (TCC), which is localized, either in renal pelvis and ureter, or in renal pelvis and urinary bladder (Stefanovic and Cosyns 2004).

Despite extensive research conducted over the past 60 years, the etiology of BEN still remains obscure and it seems that a polygenic susceptibility to the disease, in interaction with multiple environmental factors, exists (Toncheva et al. 1998; Batuman 2006). Although European Commission has adopted aristolochic acid as a cause of BEN (Cosyns 2003), still there is much evidence in favor of other environmental toxins, such as fungal mycotoxin ochratoxin A (OTA) (Castegnaro et al. 2006). However, it is still unclear whether there is a specific genetic predisposi-

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tion leading to a highly increased TCC risk in patients with BEN.

Certain nephrocarcinogenic compounds, including those associated with BEN, such as OTA, are metabolized by cytosolic glutathione S-transferase (GST) family of enzymes (Tozlovanu et al. 2012; Reljic et al. 2014). Cytosolic GSTs belong to phase II detoxifying enzymes that protect normal cells by catalyzing conjugation of electrophylic compounds of exogenous and endogenous origin with glutathione (Hayes et al. 2005). They are also an important line of defense in the cell protection from oxidative damage (Hayes et al. 2005). Based on their primary structures, cytosolic GSTs have been divided into several classes, including GSTs classes alpha (GSTA), mu (GSTM), pi (GSTP) and theta (GSTT). Common polymorphisms occur in almost all members of GSTs and several types of allelic variations have been observed. Within GSTM1 and GSTT1 class deletion polymorphism (*GSTM1-null* and *GSTT1-null*) have been described. Thus, genotypes, referred as *GSTM1-null* and *GSTT1-null,* are homozygous for the *GSTM1*0* and *GSTT1*0* alleles. Individuals with these genotypes produce no GSTM1 and GSTT1 protein and consequently have complete lack of GSTM1 and GSTT1 enzymatic activity. It has been reported that approximately 50% and 20% of Caucasian population possess *GSTM1-null* and *GSTT1-null* genotype, respectively (Landi 2000; Eaton and Bammler 1999). GSTP1 enzyme activity may be altered due to single-nucleotide polymorphism (SNP), A to G transition, which results in substitution of amino acid isoleucine (Ile) with valine (Val) (Watson et al. 1998). The relative frequencies of *GSTP1 Ile/Ile (A/A), Ile/Val (A/G) and Val/Val (G/G)* genotype are 50%, 40% and 10% in white population, respectively (Watson et al. 1998). There are three linked SNPs (G-52A, C-69T and T-567G) in proximal promoter region of *GSTA1* gene (Coles and Kadlubar 2005). This results in two *GSTA1* promoter variants which are characterized by two alleles *GSTA1*A* (-52G, -69C, -567T) and *GSTA1*B* (-52A, -69T, -567G), with differential hepatic expression (Coles and Kadlubar 2005). Namely, the nucleotide change at position -52 prevents Sp1 transcription factor binding, leading to 4-5 fold lower transcriptional activation of variant *GSTA1*B* allele (Morel et al. 2002). In Caucasians the distribution of *GSTA1AA*, *AB* and *BB* genotypes is 38%, 48% and 14%, respectively (Coles and Kadlubar 2005).

It has been shown that polymorphic expression of GSTs is associated with the altered xenobiotics detoxification efficiency, thus influencing an individual's susceptibility to carcinogens and various diseases (Di Pietro et al. 2010; Suvakov et al. 2013; Eslami and Sahebkar 2014; Kim et al. 2015). Still, there are no data regarding the association between *GST* genetic polymorphism and bladder cancer risk, neither in patients with BEN, nor in individuals who originate from BEN regions. There is evidence on the role of *GSTM1* and *GSTT1* genetic polymorphisms in the sensitivity of uroepithelial cells to genotoxic OTA effects *in vitro* (Lebrun et al. 2006). Furthermore, there are some data indicating that GSTA1 may catalyze the formation of OTA conjugates (Reljic et al. 2014).

Given the fact that GSTs are included in the metabolism of potential cause of BEN, it is very important to investigate to what extent the presence of *GST* genetic variations contributes to bladder cancer susceptibility in individuals originating from BEN region. Hence, we examined the associations between polymorphisms within the key *GST* genes and bladder cancer risk in a Serbian group of bladder cancer patients from BEN.

Materials and Methods

Study subjects

This case-control study included 201 histological confirmed TCC patients, among which 67 of them were from BEN region. A total of 122 individuals with nephrolithiasis (26 from BEN region) and no previous record of any malignant disease represented the control group. Both patients and controls were recruited from the Clinic of Urology and Nephrology, Clinical Centre of Serbia, Belgrade. All participants gave informed consent and were able to leave the study by wish. The ethical approval for study protocol was obtained by local Committee of Medical Faculty and was in compliance with the Helsinki Declaration.

Well-trained interviewers collected demographic characteristics, cigarette smoking and occupational exposure data from each participant by using a standard questionnaire, based on which two different exposure categories were defined: unexposed and exposed. The lifetime occupational exposure history included all jobs (both full and part-time jobs) in period longer than 6 months. Job description consisted of the job title, the industry or type of business, employment dates and duration, company name and location.

DNA extraction and genotyping

DNA was isolated from whole blood using commercial extraction kit (Qiagen, Inc., Chatsworth, CA, U.S.A.). *GSTM1* and *GSTT1* polymorphism were identified by PCR, while *GSTA1* and *GSTP1* were identified by restriction fragment length polymorphism PCR (RFLP-PCR) method as previously described (Suvakov et al. 2013).

Statistical analysis

Hardy-Weinberg equilibrium was tested using χ^2 test. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) in order to assess the associations of *GST* genotypes and BEN region with bladder cancer risk. All calculations were adjusted to age, gender and smoking status as potential confounders.

Results

Demographic characteristics of TCC patients and controls were presented in Table 1. No statistical significance was obtained with respect to age, gender or educational level between patients and controls. The BEN region prevalence among patients was higher than in control group (33% and 21%, respectively). We found that individuals who were from BEN region had significantly higher TCC risk then individuals originating from non-BEN region (OR = 1.84, p = 0.022). Besides, the smoking prevalence among cases was higher (74%) than in controls (54%) and occupa-

Table 1. Selected characteristics of patients with bladder cancer and controls.

Characteristic	Cases n (%)	Controls n (%)	OR (95% CI)	p
Gender				0.885
Male	114 (57)	71 (58)		
Female	87 (43)	51 (42)		
Age (years)	64.4 ± 10.1	62.7 ± 6.0		0.078
Educational level				0.646
Elementary school	62 (31)	31 (25)		
Secondary school	110 (55)	73 (60)		
University	29 (14)	18 (15)		
BEN region				
No	134 (67%)	96 (79%)	1.00	
Yes	67 (33%)	26 (21%)	1.84 (1.08-3.14)	0.022
Occupational exposure				
Yes	74 (36)	17 (14)	1.00	
No	127 (64)	105(86)	3.48 (1.88-6.39)	0.001
Smoking habits				
Never smokers	52 (26)	56 (46)	1.00	
Current smokers	149 (74)	66 (54)	2.43 (1.51-3.92)	0.001

OR, odds ratio; CI, confidence interval; BEN, Balkan endemic nephropathy.

tionally exposed individuals were more frequent among patients than controls (36% and 14%, respectively).

A significant association was observed between *GSTA1, M1* and *T1* polymorphisms, susceptibility to urinary bladder cancer and BEN region residence as presented in Table 2. Regarding *GSTA1* polymorphism, the strongest association of *GSTA1* genotype with the risk of urinary bladder cancer in individuals from BEN region residence was found in individuals with *GSTA1/AB+BB* genotype. These individuals, with lower GSTA1 expression, exhibited a 2.6 times higher bladder cancer risk compared to those with *GSTA1/AA* genotype who were from non-BEN region (OR = 2.60, p = 0.015, 95% CI = 1.20-5.50). Interestingly, similar combined effect for bladder cancer risk was observed for *GSTM1* polymorphism in individuals from BEN region. As shown, individuals with *GSTM1-active* genotype from BEN region had a 2.9 times higher risk of developing bladder cancer compared to those with the same *GSTM1* genotype from non-BEN region (OR = 2.90, p = 0.010, 95% CI = 1.30-6.60). In case of *GSTT1* genotype the strongest effect for bladder cancer risk was found in carriers of *GSTT1-active* genotype from BEN region. They exhibited 2.1-fold higher bladder cancer risk in comparison with individuals from non-BEN region and *GSTT1-active* genotype (OR = 2.10, p = 0.027, 95% CI = 1.10-4.00). Analysis of *GSTP1* polymorphism showed that carriers of *GSTP1Ile/Val+ Val/Val* genotype from BEN region had 1.9 times higher bladder cancer risk than individuals from non-BEN region with *GSTP1Ile/Ile* genotype. However, this association lacked statistical significance (OR = 1.90, p = 0.098, 95% CI = 0.90-4.20).

Discussion

The results of this study showed that low-activity *GSTA1/AB+BB,* as well as, *GSTM1* and *GSTT1-active* genotypes in combination with BEN residence contribute towards the risk of bladder cancer in Serbian TCC patients. Regarding *GSTP1* polymorphism, no association was observed between susceptibility of bladder cancer development and BEN residence.

Recently, our group showed that individuals with one or both low-activity *GSTA1*B* alleles are at 1.6-fold higher risk of BEN than individuals carrying the wild type homozygous *GSTA1*A/*A* genotype (Reljic et al. 2014). The main result obtained from this study pointed out that TCC risk in individuals from BEN areas is modified by *GSTA1* gene polymorphism. Specifically, individuals with at least one *GSTA1*B* allele who originated from BEN region had 2.6 times higher TCC risk compared to homozygous *GSTA1*A/*A* individuals who were not from this region. This is not surprising, since GSTA1 has overlapping specificity towards many different substrates (Honaker et al. 2013). Thus, GSTA1 enzyme is involved in both peroxidase and glutathione-conjugation reaction, due to which it is a key GST involved in detoxification of xenobiotics and antioxidant protection in liver. Homozygous carriers of *GSTA1* variant allele are shown to have four- to five-fold lower expression of this enzyme in liver (Coles and Kadlubar 2005). Since extensive production of reactive oxygen species leading to oxidative stress has been impli-

Table 2. Combined effect of BEN region and *GST* genotype on risk of bladder cancer.

GST / BEN region	Cases n (%)	Controls n (%)	OR (95% CI)	p
GSTA1				
AA / non-BEN	45 (23%)	38 (31%)	1.00	
AB + *BB* / non-BEN	89 (45%)	58 (48%)	1.40 (0.80-3.50)	0.232
AA / BEN	23 (11%)	11 (9%)	1.80 (0.80-4.20)	0.551
AB + *BB* / BEN	44 (21%)	15 (12%)	2.60 (1.20-5.54)	0.015
GSTM1				
**1 active*[a] / non-BEN	58 (29%)	51 (42%)	1.00	
**0 null*[b] / non-BEN	76 (38%)	45 (37%)	1.50 (0.90-2.60)	0.125
*1 *active* / BEN	32 (16%)	10 (8%)	2.90 (1.30-6.60)	0.010
*0 *null* / BEN	35 (17%)	16 (13%)	2.10 (1.00-4.44)	0.049
GSTT1				
**1 active*[a] / non-BEN	98 (48%)	72 (59%)	1.00	
**0 null*[b] / non-BEN	36 (18%)	24 (20%)	1.20 (0.60-2.20)	0.659
**1 active* / BEN	46 (23%)	16 (13%)	2.10 (1.10-4.00)	0.027
**0 null* / BEN	21 (11%)	10 (8%)	1.40 (0.60-3.20)	0.453
GSTP1				
Ile/Ile / non-BEN	57 (28%)	37 (30%)	1.00	
Ile/Val + *Val/Val* / non-BEN	77 (39%)	59 (49%)	0.84 (0.50-1.50)	0.537
Ile/Ile / BEN	27 (13%)	12 (10%)	1.40 (0.60-3.30)	0.441
Ile/Val + *Val/Val* / BEN	40 (20%)	14 (11%)	1.90 (0.90-4.20)	0.098

Adjusted for age, gender and smoking. [a]Active (present) if at least one active allele present. [b]Inactive (null) if no active alleles present.
OR, odds ratio; CI, confidence interval; GSTA1, glutathione S-transferase A1; GSTM1, glutathione S-transferase M1; GSTP1, glutathione S-transferase P1; GSTT1, glutathione S-transferase T1.

cated in tumor development, it may be suggested that liver, by its GSTA1 conjugating and peroxidase activity, plays an important role in protection against bladder carcinogens. Given a thought that GSTs catalyze some reactions of OTA metabolism, one of the possible causes of BEN, we hypothesized that *GST* polymorphisms might alter individual susceptibility to bladder cancer in individuals from BEN-region. Indeed, our previous results based on *in silico* simulation suggested that GSTA1 is involved in metabolizing OTA quinone derivatives (Reljic et al. 2014). As *GSTA1* gene polymorphism results in lowering of enzyme activity, it is reasonable to assume that these individuals will be under both increased oxidative stress and risk of cancer development when exposed to higher concentration of OTA metabolites. However, to confirm this hypothesis, further studies conducted on larger study population are necessary.

Regarding *GSTM1* polymorphism in patients with TCC originating from BEN region, our results demonstrated that individuals with *GSTM1-active* genotype are at 2.9-fold higher risk of bladder cancer compared to those from non-endemic areas. These results are in accordance with the study of Andonova et al. (2004), which reported that *GSTM1-active* genotype is more prevalent among BEN patients than in endemic control group. Similarly, Toncheva et al. (2004) have conducted another study in Bulgarian population on 95 BEN patients and 112 non-endemic controls and they have also found higher *GSTM1-active* genotype frequency in BEN patients compared to controls, but without statistical significance. Having in mind the role in detoxification, as well as, antioxidant role of GSTM1, our results are quite unexpected especially in the view of the fact that large meta-analysis unambiguously showed association between *GSTM1-null* genotype and TCC risk, independently or in association with smoking (Johns and Houlston 2000). One of the possible explanations for this result is that sometimes GST-mediated detoxification by glutathione conjugation may result in bioactivation and formation of even more toxic compounds (van Bladeren 2000). The data on potential role of GSTM1 in metabolism of OTA and aristolochic acid are scarce and inconsistent.

Interestingly, *GSTM1 is* not the only active form of GST associated with TCC risk in patients originating from BEN region. Our results also shown that BEN-area residents carriers of *GSTT1-active* genotype have two times higher risk to develop TCC of urinary bladder in comparison to non-BEN-area residents with the same genotype. These findings may be explained by the well-known role of

GSTT1 in bioactivation of various cancerogenic compounds, such as trichloroethylene (Moore et al. 2010). Indeed, our group has showed that *GSTT1-active* genotype is associated with TCC risk in men exposed to pesticides (Matic et al. 2014). Regarding the potential role of GSTT1 in metabolism of OTA and aristolochic acid, the results of our recent investigation did not show involvement of this enzyme in OTA metabolism (Reljic et al. 2014), while data on aristolochic-acid metabolism and GSTT1 are lacking. Still, it is noteworthy mentioning that our results on association of *GSTT1* polymorphism between risk of TCC bladder cancer and residence in BEN-area are in line with results of Lebrun et al. (2006) who pointed out the role of *GSTT1* polymorphism in the extent of DNA damage induced by OTA. Namely, when uroepithelial cells from different human donors were treated with OTA, in cells with *GSTT1-active* genotype the DNA damage was more extensive than in those with *GSTT1-null* genotype.

In conclusion, the results obtained from this study may improve our understanding of the potential role of *GST* polymorphism on TCC risk in BEN region residents. However, there are certain limitations that should be considered. The first one is the relatively small sample size that is in favor of creating potential biases. The second one is our hospital-based control group that may not reflect the general population characteristics. Although we adjusted all results by age, gender and smoking status, there are probably more confounding factors that might influence the results. Therefore, further investigations with larger sample size and more rigorous designs are needed for further elucidation of the role of *GST* polymorphisms in relation to BEN and bladder cancer risk.

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Conflict of Interest

We declare no conflict of interest.

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