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Concomitance of Polymorphisms in Glutathione Transferase Omega Genes Is Associated with Risk of Clear Cell Renal Cell Carcinoma

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Glutathione S-transferases (GSTs), a superfamily of multifunctional enzymes, play an important role in the onset and progression of renal cell carcinoma (RCC). However, novel GST omega class (GSTO), consisting of GSTO1-1 and GSTO2-2 isoenzymes, has not been studied in RCC yet. Two coding single nucleotide polymorphisms (SNPs) supposedly affect their functions: *GSTO1**C419A (rs4925) causing alanine to aspartate substitution (*A140D) and *GSTO2**A424G (rs156697) causing asparagine to aspartate substitution (*N142D), and have been associated with several neurodegenerative diseases and cancers. Functional relevance of yet another *GSTO2* polymorphism, identified at the 5′ untranslated (5′UTR) gene region (*GSTO2**A183G, rs2297235), has not been clearly discerned so far. Therefore, we aimed to assess the effect of specific *GSTO1* and *GSTO2* gene variants, independently and in interaction with established risk factors (smoking, obesity and hypertension) on the risk for the most aggressive RCC subtype, the clear cell RCC (ccRCC). Genotyping was performed in 239 ccRCC patients and 350 matched controls, while plasma levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, were determined by ELISA. As a result, combined effect of all three variant genotypes exhibited almost 3-fold risk of RCC development. Additionally, this association was confirmed at the haplotype level [variant *GSTO1**A/*GSTO2**G (rs156697)/*GSTO2**G (rs2297235) haplotype], suggesting a potential role of those variants in propensity to RCC. Regarding the gene-environment interactions, variant *GSTO2**G (rs156697) homozygous smokers are at higher ccRCC risk. Association in terms of oxidative DNA damage was found for *GSTO2* polymorphism in 5′UTR and 8-OHdG. In conclusion, the concomitance of GSTO polymorphisms may influence ccRCC risk.

Keywords: *GSTO1*; *GSTO2*; oxidative DNA damage; polymorphism; renal cell carcinoma
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Introduction

Glutathione S-transferases (GSTs), a large family of multifunctional enzymes, play a significant role in the onset and progression of renal cell carcinoma (RCC) (Pljesa-Ercegovac et al. 2008; Searchfield et al. 2011). Cytosolic GSTs are involved in detoxification of electrophilic metabolites of endogenous or exogenous origin by conjugation with glutathione (Strange et al. 2001). A vast number of carcinogens implicated in the occurrence of RCC, such as polycyclic aromatic hydrocarbon diol-epoxides and halogenated solvents (Sweeney et al. 2000), as well as chemotherapeutic agents are metabolized by GSTs (Wu and Dong 2012). Recent studies suggest that the possible mechanisms underlying the association of GSTs with cancer development and progression might be contributed to their role in cellular redox regulation (Tew and Townsend 2012). It is noteworthy to mention that concerning redox status, RCC belongs to a group of solid tumors with a certain level of oxidative distress. Specifically, prooxidative environment might be important in the early phase of RCC development, while in the course of cancer progression, a shift towards more reduced state occurs (Lusini et al. 2001). Lately, other biologically important roles of these enzymes have

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been specified, including protein-protein interactions. Namely, GSTs act as modulators of the mitogen-activated protein kinase (MAPK) signaling pathway involved in cellular survival and apoptosis (Tew and Townsend 2012). Furthermore, polymorphisms in GST genes could affect both individual response to carcinogen exposure and pharmacogenomic-based cancer treatment (Lo and Ali-Osman 2007). So far the association of common GST gene polymorphisms *(GSTM1*, *GSTT1*, *GSTP1* and *GSTA1)*, independently or in interaction with environmental factors, with increased risk of developing RCC has been shown (Coric et al. 2016; Zhong et al. 2018). Novel omega class GST consists of two isoenzymes, GSTO1-1 and GSTO2-2. In contrast to all other GSTs, they exhibit the whole range of specific activities, including a thioltransferase, dehydroascorbate reductase and monomethylarsonat reductase activity (Board and Menon 2016). In this line, both enzymes are considered to be involved in the regulation of cellular redox balance: GSTO1-1 by its deglutathionylation activity and GSTO2-2 as the enzyme with the highest dehydroascorbate-reductase (DHAR) activity that is responsible for maintaining high cellular vitamin C levels. GSTO1-1 also possesses numerous regulatory roles, such as modulation of ryanodine receptors, posttranslational processing of interleukin-1β (IL1-β) and proposed anti-apoptotic role (Dulhunty et al. 2001; Laliberte et al. 2003; Piaggi et al. 2010). Furthermore, it has been suggested that GSTO1-1 could also contribute to tumor chemoresistance. As shown for cisplatin-resistant sublines of ovarian cancer and HeLa cells, increased GSTO1-1 expression was associated with the activation of survival signaling pathways (Akt and ERK1/2) and inhibition of apoptotic MAPK pathway (JNK1) (Yan et al. 2007; Piaggi et al. 2010). Numerous studies which investigated apoptosis and survival pathways in terms of RCC (Banumathy and Cairns 2010) have confirmed the therapeutic potential of phosphatidylinositol-3-Kinase (PI3K)/Akt inhibitors (Lin et al. 2006; Zhong et al. 2016). In that context, it seems that this novel class of GST enzymes could contribute to RCC progression, affecting both the proliferation capacity of tumor cells and response to therapy.

Two GSTO actively transcribed genes (*GSTO1* and *GSTO2)* were identified in the human population (Fig. 1), located 1.5 kb apart on the long arm of chromosome 10 (10q25.1) (Board and Menon 2016). Mukherjee et al. (2006) decribed a total of 31 polymorphisms in *GSTO1* and 66 polymorphisms of *GSTO2* gene, two of which are commonly studied: *GSTO1**C419A (rs4925) causing alanine to aspartate substitution in amino acid 140 (*A140D) and *GSTO2**A424G (rs156697) which causes an asparagine to aspartate substitution in amino acid 142 (*N142D). Moreover, in recent years several studies investigated another polymorphism of *GSTO2,* identified within the 5′ untranslated (5′UTR) gene region (*GSTO2**A183G, rs2297235) (Wang et al. 2009). Strong linkage disequilibrium has been demonstrated for all three mentioned polymorphisms (Wang et al. 2009). Moreover, it has been shown that the most investigated *GSTO1* polymorphism leads to change in aforementioned deglutathionylase and thioltransferase activity, however, without affecting their monomethylarsonat reductase activity (Tanaka-Kagawa et al. 2003; Whitbread et al. 2003; Menon and Board 2013). Regarding *GSTO2* rs156697 polymorphism, a strong association was indicated between variant *GSTO2*G* allele and its protein levels (Mukherjee et al. 2006; Allen et al. 2012). Therefore, it came as no surprise that polymorphisms of GST omega class members have been investigated in regard to a number of clinical disorders, including Alzheimer's disease, Parkinson's disease, vascular dementia and stroke, amyotrophic lateral sclerosis, chronic obstructive pulmonary disease (Board and Menon 2016) and cancer (Xu et al. 2014). Although the particular association of these polymorphisms with several neurodegenerative diseases might be explained by modulating role of GSTO1-1 and GSTO2-2 in redox balance regulation (Allen et al. 2012; Board and Menon 2016), their functional relevance in cancer has not been clearly discerned (Xu et al. 2014).

Due to observed expression of GST omega class enzymes in kidney parenchyma (Whitbread et al. 2005), we hypothesized that *GSTO1* and *GSTO2* polymorphisms

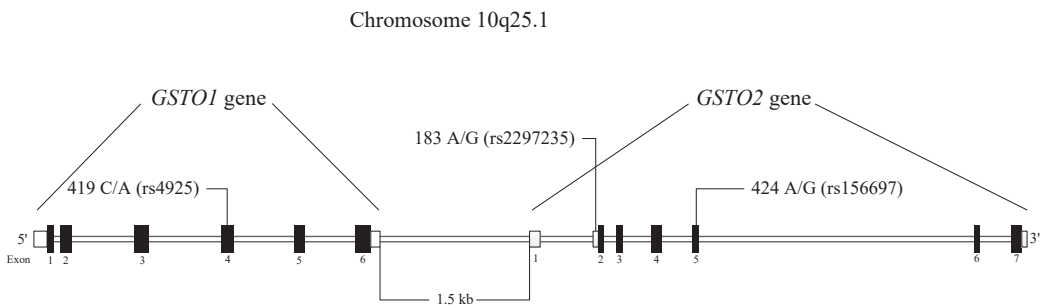

Fig. 1. Schematic representation of the investigated polymorphisms in *GSTO1* and *GSTO2* genes.
GSTO1 and *GSTO2* genes are located 1.5 kb apart on the long arm of chromosome 10 (10q25.1). Exons that encode the open read frame are represented as black rectangles, whereas white rectangles represent untranslated regions (UTRs). The SNPs are indicated as position with the allele variants followed by the rs number. These polymorphisms are in strong linkage disequilibrium.

could either alone or in association with known risk factors (smoking, obesity and hypertension) influence the risk of clear cell RCC (ccRCC), the most aggressive RCC subtype. Therefore, in this study, we for the first time evaluated the modifying effect of three specific *GSTO1* and *GSTO2* gene variants (*GSTO1**C419A rs4925, *GSTO2**A424G rs156697 and *GSTO2**A183G rs2297235), independently and in interaction with known risk factors, on propensity to develop ccRCC. To obtain a clear insight into the functional significance of these findings, we also studied the association of distinct *GSTO1* and *GSTO2* polymorphisms with the systemic level of oxidative DNA damage, determined as the level of 8-OHdG.

Materials and Methods

Study population

We enrolled 239 patients (162 men, 77 women; average age 58.94 ± 11.64 years) from the Urology Clinic, Clinical Center of Serbia, Belgrade, diagnosed with ccRCC that was histologically confirmed by pathologists specialized in uropathology. The control group comprised 350 sex- and age-matched individuals (217 men, 133 women; average age 60.16 ± 11.11 years) recruited at the same clinical center, with no previous personal history of cancer. Upon obtaining informed consent, each subject was interviewed using a standard questionnaire, composed at the Institute of Epidemiology, University of Belgrade Faculty of Medicine (UBFM). The information regarding demographic characteristics, as well as known RCC risk factors (smoking history, hypertension and obesity) were collected.

Ethical approval

All procedures performed in the study were in accordance with the ethical standards of the 1964 Declaration of Helsinki revised in 2013. The study was approved by the Institutional Ethical board (October13, 2011, approval number 29/X-3, Faculty of Medicine, University of Belgrade, Serbia).

Informed consent

Informed consent was obtained from all individual participants included in the study.

GST genotyping

DNA from the whole blood was isolated using QIAamp DNA mini kit (Qiagen,USA). *GSTO1**C419A (rs4925), *GSTO2**A424G (rs156697) and *GSTO2**A183G (rs2297235) polymorphisms were determined by quantitative polymerase chain reaction (qPCR), performed on Mastercycler ep realplex (Eppendorf, Germany) using Applied Biosystems TaqMan SNP Genotyping assay (Thermo Fisher Scientific, USA, assay ID: C_11309430_30, C_3223136_1 and C_3223142_1, respectively).

Determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG)

The quantification of 8-OHdG (ng/ml) in plasma samples of ccRCC patients and controls was performed using the OxiSelect Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc., USA).

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Selected characteristics among ccRCC patients and controls were compared using the $\chi 2$ test for categorical variables, whereas depending on data distribution, the Student's t test or Mann-Whitney ranksum test was used for continuous variables. Distribution was tested by using graphical methods, as well as Kolmogorov-Smirnov and Shapiro-Wilk tests. $\chi 2$ test was used to test deviation of the genotype distribution from Hardy-Weinberg equilibrium. Estimation of linkage disequilibrium (LD) between pairs of SNPs was examined using the SNPStats (Solé et al. 2006). The strength of LD was expressed in terms of $D' = D/Dmax$. Effects of genetic variants on ccRCC risk were computed by odds ratios (OR) and 95% confidence intervals (CI) using logistic regression analysis adjusted for age, sex, smoking, hypertension and obesity.

Results

Baseline characteristics of 239 patients with ccRCC and 350 controls are shown in Table 1. There was no significant difference between patients and controls with regards to age, sex, smoking and obesity ($P > 0.05$). We found that hypertensive subjects were at 3.54-fold higher risk of developing ccRCC compared to normotensive subjects (95% CI: 2.35-5.32, $P < 0.001$).

Distribution of the *GSTO1* (rs4925) and *GSTO2* (rs156697, rs2297235) genotypes in patients and controls is presented in Table 2. As shown, carriers of *GSTO1**A/A and *GSTO2**G/G (rs156697) variant genotypes were at higher risk of ccRCC development when compared to referent genotypes, but this association did not reach statistical significance (OR = 1.35, 95% CI: 0.70-2.61, P = 0.364 and OR = 1.78, 95% CI: 0.91-3.50, P = 0.092, respectively). However, when *GSTO1**C419A and *GSTO2**A424G polymorphisms were analyzed in combination, the significant association was obtained. Namely, individuals with combined variant *GSTO1**A/A and *GSTO2**G/G (rs156697) genotypes had 2.6-fold higher risk of developing cancer compared to referent genotype combination (95% CI: 1.09-6.19, P = 0.031). Combined effect of all three polymorphisms showed no further increase in the risk of ccRCC development (Table 2). It is important to note that our results on combined effects of GSTO polymorphisms were also confirmed in haplotype analysis. Namely, since both *GSTO1* and *GSTO2* genes are located on the same chromosome, just 1.5 kb apart, we performed a linkage disequilibrium analysis. In this LD analysis, we found D' of 0.64 for *GSTO1**C419A and *GSTO2**A424G ($P < 0.001$), 0.83 for *GSTO1**C419A and *GSTO2**A183G ($P < 0.001$), and 0.80 for *GSTO2**A424G and *GSTO2**A183G ($P < 0.001$), indicating high LD between these SNPs. The most prevalent haplotype among controls (52%) and patients (56%) is H1, consisting of *GSTO1**C, *GSTO2**A (rs156697) and *GSTO2**A (rs2297235) wild-type alleles. Carriers of H2 haplotype, represented by one copy of variant *GSTO1**A, *GSTO2**G (rs156697) and *GSTO2**G (rs2297235), had significantly increased risk of 1.46 for ccRCC (95% CI: 1.02-2.09, P = 0.041) (Table 3).

Table 1. Baseline characteristics of ccRCC patients and controls.

	Controls	Patients	OR (95% CI)	P
Age (years)[a]	60.16 ± 11.11	58.94 ± 11.64		0.207
Sex, n (%)				
Male	217 (62)	162 (68)	1.00	
Female	133 (38)	77 (32)	1.20 (0.79-1.84)[b]	0.391
Smoking, n (%)				
Never	164 (49)	80 (41)	1.00	
Ever[c]	173 (51)	114 (59)	1.50 (0.99-2.26)[d]	0.057
Pack-years[e]	30.00 (1.00-120.00)	31.25 (0.30-141.00)		0.267
Hypertension, n (%)				
No	232 (71)	89 (45)	1.00	
Yes	96 (29)	109 (55)	3.54 (2.35-5.32)[f]	< 0.001
Obesity, n (%)				
BMI < 30	253 (83)	157 (80)	1.00	
BMI ≥ 30 [g]	50 (17)	39 (20)	1.09 (0.66-1.81)[h]	0.732
BMI (kg/m^2)[a]	26.51 ± 3.83	26.65 ± 4.41		0.710
Fuhrman grade, n (%)[i]				
Grade I	30 (15)			
Grade II	112 (55)			
Grade III	52 (26)			
Grade IV	8 (4)			
pT stage, n (%)[i]				
pT1	100 (45)			
pT2	24 (11)			
pT3	94 (42)			
pT4	5 (2)			

[a]mean ± SD.
[b]OR, odds ratio adjusted to age, smoking status, hypertension, obesity.
[c]Every day smoking during a minimum of 60-day period prior to the study onset.
[d]OR, odds ratio adjusted to age, sex, hypertension, obesity.
[e]Median (min-max).
[f]OR, odds ratio adjusted to age, sex, smoking status, obesity.
[g]BMI, body mass index; Obese participants were defined as individuals with BMI above 30.
[h]OR, odds ratio adjusted to age, sex, smoking status, hypertension; CI, confidence interval.
[i]Available data on patients' tumor grade and stage, depending on the type of surgery and pathohistology diagnostics.
P < 0.05 was considered to be statistically significant.

We also investigated possible modifying effect of GSTO genotypes in conjunction with hypertension, obesity and smoking, as recognized risk factors, on ccRCC risk. We found no modifying effect of GSTO genotypes with hypertension (Table 4) and obesity (Table 5), confirming the significance of hypertension as ccRCC independent risk factor, regardless of GSTO genotypes. However, significant modifying effect on ccRCC risk conferred by smoking has been found only in *GSTO2**G/G (rs156697) carriers (OR = 2.44, 95% CI: 1.04-5.71, P = 0.040) (Table 6), while another two studied polymorphisms (*GSTO1* rs4925 and *GSTO2* rs2297235) did not show any association with smoking.

Regarding the degree of oxidative DNA damage in plasma samples, 8-OHdG levels were higher in ccRCC patients compared to controls (1.00 ng/ml vs. 0.70 ng/ml, respectively). In attempt to discern functional role of *GSTO1* and *GSTO2* polymorphisms, we assessed the degree

Table 2. Distribution of individual *GSTO1* (rs4925) and *GSTO2* (rs156697 and rs2297235), as well as combined *GSTO1/GSTO2* genotypes in ccRCC patients and controls.

Genotype	Controls, n (%)	Patients, n (%)	OR (95% CI)[a]	P
***GSTO1* rs4925[b]**				
*C/C (wild-type)	128 (38)	89 (38)	1.00	
*C/A (heterozygote)	169 (50)	116 (49)	0.87 (0.56-1.33)	0.512
*A/A (variant)	41 (12)	31 (13)	1.35 (0.70-2.61)	0.364
***GSTO2* rs156697[c]**				
*A/A (wild-type)	149 (45)	92 (38)	1.00	
*A/G (heterozygote)	148 (44)	119 (50)	1.26 (0.83-1.92)	0.283
*G/G (variant)	36 (11)	28 (12)	1.78 (0.91-3.50)	0.092
***GSTO2* rs2297235[d]**				
*A/A (wild-type)	163 (48)	97 (42)	1.00	
*A/G (heterozygote)	133 (39)	111 (48)	1.27 (0.84-1.94)	0.263
*G/G (variant)	42 (12)	23 (10)	1.06 (0.54-2.06)	0.871
Combined *GSTO1* rs4925/*GSTO2* rs156697				
*CC+*CA/ *AA+*AG	267 (82)	197 (84)	1.00	
*CC+*CA/ *GG	17 (5)	8 (3)	0.77 (0.29-2.04)	0.602
*AA/ *AA+*AG	22 (7)	12 (5)	0.85 (0.37-1.97)	0.709
*AA / *GG	18 (6)	19 (8)	2.60 (1.09-6.19)	0.031
Combined *GSTO1* rs4925/*GSTO2* rs2297235				
*CC+*CA/ *AA+*AG	277 (85)	195 (85)	1.00	
*CC+*CA/ *GG	9 (3)	3 (1)	0.41 (0.10-1.63)	0.205
*AA/ *AA+*AG	10 (3)	11 (5)	1.75 (0.63-4.89)	0.283
*AA / *GG	31 (9)	20 (9)	1.27 (0.62-2.59)	0.517
Combined *GSTO2* rs156697/*GSTO2* rs2297235				
*AA+*AG / *AA+*AG	276 (85)	200 (86)	1.00	
*AA+*AG / *GG	14 (4)	4 (2)	0.39 (0.12-1.27)	0.118
*GG / *AA+*AG	11 (3)	8 (4)	1.20 (0.42-3.45)	0.741
*GG / *GG	25 (8)	19 (8)	1.55 (0.72-3.37)	0.264
Combined *GSTO1* rs4925/*GSTO2* rs156697/ *GSTO2* rs2297235				
*CC+*CA/ *AA+*AG/ *AA+*AG	259 (81)	187 (82)	1.00	
*AA / *GG/ *GG	18 (6)	19 (8)	2.57 (1.08-6.10)	0.033

[a]OR, odds ratio adjusted to age, sex, smoking status, hypertension, obesity; CI, confidence interval.
[b]For *GSTO1* rs4925, genotyping was successful in 236 of 239 patients and 338 of 350 controls.
[c]For *GSTO2* rs156697, genotyping was successful in all recruited patients and 333 of 350 controls.
[d]For *GSTO2* rs2297235, genotyping was successful in 231 of 239 patients and 338 of 350 controls.
$P < 0.05$ was considered to be statistically significant.

of oxidative DNA damage in ccRCC patients stratified according to GSTO genotype. The results obtained showed that levels of 8-OHdG, a biomarker of oxidative DNA damage, were significantly higher in patients with *GSTO2**G/G variant genotype (rs2297235) than in carriers of at least one *GSTO2**A referent allele (1.41 ng/ml vs. 0.99 ng/ml, respectively $P = 0.042$). No association was found with plasma 8-OHdG levels and *GSTO1* or another *GSTO2* (rs156697) genotypes (Table 7).

Discussion

The results of this study have shown that individuals with combined variant *GSTO1* and *GSTO2* genotypes exhibit higher risk of ccRCC development compared to the carriers of both referent genotypes. Regarding the gene-environment interactions and RCC risk, variant *GSTO2*

Table 3. Haplotypes of *GSTO1* (rs4925) and *GSTO2* (rs156697 and rs2297235) in relation to the risk of ccRCC.

Haplotype	*GSTO1* rs4925	*GSTO2* rs156697	*GSTO2* rs2297235	Controls, %	Patients, %	OR (95% CI)[a]	P
H1	*C	*A	*A	52	56	1.00	
H2	*A	*G	*G	22	31	1.46 (1.02-2.09)	0.041
H3	*A	*A	*A	9	5	0.55 (0.31-0.99)	0.047
H4	*C	*G	*A	6	5	0.95 (0.49-1.86)	0.880
H5	*A	*A	*G	5	1	0.23 (0.08-0.68)	0.008
H6	*C	*G	*G	4	1	0.06 (0.01-0.72)	0.027

Global haplotype association P-value: < 0.0001.
[a]OR, odds ratio adjusted to age, sex, smoking status, hypertension, obesity; CI, confidence interval.
P < 0.05 was considered to be statistically significant.

Table 4. Distribution of *GSTO1* (rs4925) and *GSTO2* (rs156697 and rs2297235) genotypes in relation to hypertension in ccRCC patients and controls.

	Controls, n (%)	Patients, n (%)	OR (95% CI)[a]	P
Combined *GSTO1* rs4925/hypertension				
*CC+*CA/no	194 (61)	71 (36)	1.00	
*CC+*CA/yes	86 (27)	98 (50)	3.96 (2.54-6.16)	< 0.001
*AA/no	30 (9)	15 (8)	1.95 (0.94-4.07)	0.074
*AA /yes	9 (3)	11 (6)	3.61 (1.34-9.67)	0.011
Combined *GSTO2* rs156697/ hypertension				
*AA+*AG /no	196 (62)	75 (38)	1.00	
*AA+*AG /yes	86 (27)	99 (50)	3.83 (2.47-5.93)	< 0.001
*GG/no	25 (8)	14 (7)	2.10 (0.98-4.52)	0.057
*GG/yes	8 (3)	10 (5)	3.63 (1.28-10.26)	0.015
Combined *GSTO2* rs2297235/ hypertension				
*AA+*AG/no	196 (61)	75 (39)	1.00	
*AA+*AG/yes	83 (26)	97 (51)	3.80 (2.45-5.89)	< 0.001
*GG/no	30 (10)	10 (5)	1.29 (0.57-2.91)	0.543
*GG/yes	11 (3)	10 (5)	2.43 (0.93-6.29)	0.069

[a]OR, odds ratio adjusted to age, sex, smoking status, obesity; CI, confidence interval.
P < 0.05 was considered to be statistically significant.

homozygous smokers are at higher risk when compared to non-smokers with at least one referent allele. Association in terms of oxidative phenotype was found only for *GSTO2* polymorphism in 5′UTR and 8-OHdG.

Until now, significant association between *GSTO1**C419A polymorphism (rs4925) and risk of acute lymphoblastic leukemia, hepatocellular, breast, bile duct and non-small cell lung cancer has been reported (Xu et al. 2014), whereas *GSTO2**A424G polymorphism (rs156697) increased the risk of ovarian (Pongstaporn et al. 2006), breast (Xu et al. 2014) and bladder cancer (Djukic et al. 2015). In line with these findings, we also showed that *GSTO2**A424G polymorphism (rs156697) increases the risk of ccRCC, but the effect was statistically significant when it was analyzed in combination with *GSTO1* polymorphism or smoking. Considering the observed significant LD of these genes, we also focused on evaluation of possible effect of haplotypes on ccRCC risk. We found that the carriers of H2 haplotype, comprising *GSTO1**A (rs4925), *GSTO2**G (rs156697) and *GSTO2**G (rs2297235) allelic variants, are at increased risk for ccRCC.

In the course of RCC development, complex changes of cellular redox regulation contribute to survival of tumor cells and disease progression (Pljesa-Ercegovac et al. 2008). Based on specific set of antioxidant and regulatory activities of the GSTO enzymes, the polymorphisms of

Table 5. Distribution of *GSTO1* (rs4925) and *GSTO2* (rs156697 and rs2297235) genotypes in relation to obesity in ccRCC patients and controls.

	Controls, n (%)	Patients, n (%)	OR (95% CI)[a]	P
Combined *GSTO1*/obesity[b]				
*CC+*CA/non-obese	213 (73)	134 (69)	1.00	
*CC+*CA/obese	48 (16)	34 (18)	1.02 (0.60-1.74)	0.948
*AA/ non-obese	29 (10)	20 (10)	1.31 (0.68-2.52)	0.413
*AA / obese	2 (1)	5 (3)	3.74 (0.65-21.60)	0.140
Combined *GSTO2* rs156697/obesity				
*AA+*AG /non-obese	216 (75)	136 (69)	1.00	
*AA+*AG /obese	46 (16)	37 (19)	1.14 (0.67-1.93)	0.624
*GG/ non-obese	25 (9)	21 (11)	1.67 (0.86-3.25)	0.130
*GG/obese	3 (1)	2 (1)	1.00 (0.13-7.87)	0.997
Combined *GSTO2* rs2297235/ obesity				
*AA+*AG /non-obese	214 (72)	136 (72)	1.00	
*AA+*AG /obese	49 (16)	34 (18)	0.94 (0.56-1.61)	0.831
*GG/non-obese	32 (11)	15 (8)	0.79 (0.40-1.56)	0.489
*GG/obese	1 (1)	4 (2)	4.05 (0.42-39.57)	0.229

[a]OR, odds ratio adjusted to age, sex, smoking status, hypertension; CI, confidence interval.
[b]Obese participants were defined as individuals with BMI (body mass index) above 30.
P < 0.05 was considered to be statistically significant.

Table 6. Distribution of *GSTO1* (rs4925) and *GSTO2* (rs156697 and rs2297235) genotypes in relation to smoking status in ccRCC patients and controls.

	Controls, n (%)	Patients, n (%)	OR (95% CI)[a]	P
Combined *GSTO1* rs4925/smoking[b]				
*CC+*CA/ non-smokers	140 (43)	67 (35)	1.00	
*CC+*CA/smokers	149 (46)	97 (51)	1.55 (0.99-2.42)	0.053
*AA/ non-smokers	17 (5)	12 (6)	1.91 (0.74-4.90)	0.182
*AA /smokers	20 (6)	15 (8)	1.91 (0.85-4.30)	0.116
Combined *GSTO2* rs156697/smoking				
*AA+*AG / non-smokers	142 (44)	70 (36)	1.00	
*AA+*AG / smokers	151 (46)	99 (51)	1.51 (0.97-2.35)	0.067
*GG/ non-smokers	16 (5)	9 (5)	1.46 (0.54-3.97)	0.460
*GG/smokers	15 (5)	15 (8)	2.44 (1.04-5.71)	0.040
Combined *GSTO2* rs2297235/smoking				
*AA+*AG/ non-smokers	138 (42)	71 (37)	1.00	
*AA+*AG/ smokers	153 (47)	100 (52)	1.45 (0.94-2.25)	0.095
*GG/ non-smokers	21 (6)	8 (4)	0.86 (0.32-2.31)	0.762
*GG/smokers	17 (5)	12 (6)	1.40 (0.59-3.33)	0.447

[a]OR, odds ratio adjusted to age, sex, hypertension, obesity; CI, confidence interval.
[b]Smoking status was categorized into non-smokers and smokers with respect to the limit of a minimum of 60-day period of every day smoking prior to their enrollment in the study.
P < 0.05 was considered to be statistically significant.

Table 7. The association between GSTO genotypes and the levels of 8-OHdG in ccRCC patients.

Genotype	8-OHdG in ccRCC patients (ng/ml)[a]	P
***GSTO1* rs4925**		
*C/C+*C/A	0.99 (0.39-1.80)	
*A/A	1.21 (0.92-1.61)	0.154
***GSTO2* rs156697**		
*A/A+*A/G	1.00 (0.39-1.80)	
*G/G	1.09 (0.62-1.61)	0.448
***GSTO2* rs2297235**		
*A/A+*A/G	0.99 (0.39-1.62)	
*G/G	1.41 (0.92-1.80)	0.042

[a]Median (min-max).
$P < 0.05$ was considered to be statistically significant.

GSTO1 and *GSTO2* might influence inter-individual susceptibility to oxidative stress. It has been shown that *GSTO1**C allelic variant has higher deglutathionylation and thioltransferase activity in contrast to *A variant (Tanaka-Kagawa et al. 2003; Menon and Board 2013). Since glutathionylation, as post-translational modification, can influence the activity of many proteins involved in tumor growth, the presence of *GSTO1* allelic variants with altered activity could provide a plausible mechanism to explain the associations between this genetic polymorphism and different cancers (Xu et al. 2014). In addition to *GSTO1*, it seems that polymorphisms in *GSTO2* could also affect primarily its antioxidant dehydroascorbate reductase (DHAR) activity (Whitbread et al. 2005; Piacentini et al. 2013), which is responsible for regeneration of ascorbic acid (vitamin C). Moreover, increased uptake of dehydroascorbate via the GLUT1 transporters has been shown for some colorectal tumor cell lines (Yun et al. 2015). Since increased expression of GLUT1 transporters is common feature of different solid tumors, including RCC, this phenomenon of higher dehydroascorbate uptake needs further elucidation. Nevertheless, low DHAR activity in subjects with both variant *GSTO2* alleles might result in deficient vitamin C regeneration and accumulation of oxidized form, dehydroascorbate, contributing to disruption of redox homeostasis.

An interesting approach to study relevance of antioxidant gene polymorphisms is to compare the level of oxidative stress byproducts in individuals stratified according to their antioxidant gene variants. In the study of oxidative DNA modifications among malignant diseases, 8-OHdG seems to be the most appropriate biomarker. Indeed, bladder cancer patients, carriers of *GSTM1*-null and *GSTA1-variant* genotypes, exhibited higher urinary 8-OHdG levels (Savic-Radojevic et al. 2013). In this line, our results on the higher levels of 8-OHdG, a biomarker of free radical-induced DNA-oxidative damage, among ccRCC patients with *GSTO2**G/G variant genotype (rs2297235) seem plausible. Taken together, the presence of H2 haplotype, comprising low deglutathionylation and low DHAR activity, might underlie the impaired redox regulation and increased RCC risk. Regarding joint effect of GST polymorphisms and smoking on ccRCC risk, it seems that smoking, as an important source of ROS (Valavanidis et al. 2009), contributes to genotype-associated ccRCC risk in carriers of *GSTO2*-variant genotype. Indeed, we found that smokers carriers of *GSTO2**G/G (rs156697) were at almost 2.5-fold increased RCC risk.

Potential interplay between disrupted redox homeostasis, hypoxia/pseudohypoxia, diminished carcinogenic detoxification and the hypoxia inducible factor (HIF) signaling pathway emerged as important aspect of RCC pathogenesis. Interestingly, oxygen-dependent protein hydroxylases, which represent main regulators of HIF activity by marking HIFα for ubiquitinylation and consequent proteasomal degradation, use Fe(II), 2-oxoglutarate and vitamin C as cofactors. In that context, it might be speculated that vitamin C-dependent inhibition of the HIF pathway may provide additional approach for controlling tumor progression and inflammation (Li and Schellhorn 2007). Inefficient regeneration of vitamin C, as a potential result of *GSTO2* polymorphism, might affect HIFα hydroxylation, resulting in HIFα stabilization and downstream overexpression of genes involved in angiogenesis, proliferation, cell migration and invasion, metabolic shift towards glycolysis, survival, erythropoiesis, which eventually contribute to ccRCC tumorigenesis (Mehdi and Riazalhosseini 2017). Recently, HIF-α inhibitors are proposed as potential therapeutic strategy in ccRCC after significant results in inhibiting tumor growth and angiogenesis (Wallace et al. 2016).

Assuming the specific roles of GSTO enzymes in these processes, our results support the hypothesis that GSTO polymorphisms might be associated to the risk of ccRCC, with special emphasis on *GSTO2*-variant genotype. In the future, investigations of these polymorphisms in conjunction with exposure to specific environmental factors, known

to be associated to RCC development, conducted on larger cohort of patients would be warranted.

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Conflict of Interest

The authors declare no conflict of interest.

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