Association of AKR1C3 Polymorphisms with Bladder Cancer

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Purpose: Polymorphisms in the genes coding for the carcinogen metabolizing enzymes may affect enzyme activities and alter the activation and detoxification rates of the carcinogens. AKR1C3 is one of the very polymorphic xenobiotic metabolizing enzymes involved in the bioactivation process. Here we aimed to investigate the association of two single nucleotide polymorphisms in AKR1C3, rs12529 (c.15C > G) and rs1937920 (12259 bp 3' of STP A > G) with urinary bladder cancer (UBC).

Materials and Methods: Two-hundred fifty UBC cases and 250 control subjects were genotyped using the Polymerase Chain Reaction and Restriction Fragment Length method. Associations of the genotypes with UBC risk and tumor characteristics were assessed using logistic regression and Fisher's exact test. The results are corrected for multiple testing.

Results: We identified strong associations between the studied AKR1C3 variants and UBC risk. The homozygous variant genotype of rs12529 was found to be inversely associated with UBC, and rs1937920 was shown to be associated with increased risk of UBC. None of the genotypes were found to be significantly associated with tumor characteristics.

Conclusion: We provided evidence that rs12529 and rs1937920 are significant in the molecular pathogenesis of UBC. However, the results presented here should be regarded as preliminary and might represent a first step of future larger studies aiming to better elucidate the role of AKR1C3 polymorphisms in the susceptibility to bladder cancer.

Keywords: genetic association studies; humans; polymorphism; single nucleotide; urinary bladder neoplasms; genetics; 3-hydroxysteroid dehydrogenases; aryl hydrocarbon hydroxylases; aryl hydrocarbon receptor nuclear translocator.

INTRODUCTION

Urinary Bladder Cancer (UBC) is the ninth most common cancer worldwide, with approximately 400.000 new cases each year.[1] Despite increased awareness against certain risk factors and recent promising developments in the diagnosis and treatment, worldwide incidence of UBC increases steadily. [2] Bladder cancer is three to five times higher in men than women, and its incidence increases with age for both sexes, peaking at the seventh decade.[3]. The main molecular pathways leading to UBC development are altered xenobiotic metabolism and mutations in genes involved in DNA repair, tumor suppression and cell proliferation mechanisms.[4-8] The most common risk factor for UBC is cigarette smoking, followed by occupational exposure to aromatic amines used in the production of dyes, rubber and textiles and to polycyclic aromatic hydrocarbons (PAHs) formed during combustion of fossil and carbon-containing fuels.[9,10] These well-studied and mutagenic carcinogens should undergo bioactivation in order to exert their carcinogenic potential. Alterations in the activation and following detoxification mechanisms of these carcinogenic chemicals result in the formation of DNA adducts, which eventually lead to DNA damage. This process constitutes the basis for genetic susceptibility to UBC.[4] Therefore, studies investigating genetic susceptibility to UBC focus on genes involved in xenobiotic metabolism and DNA repair. The ultimate function of the xenobiotic metabolism is detoxification of carcinogens, where chemical modifications catalyzed by Phase I enzymes result in the activation of carcinogens, and those catalyzed by Phase II enzymes facilitate the excretion of these compounds. Polymorphisms in the genes coding for the xenobiotic enzymes may induce altered enzyme activities that confer susceptibility to various cancers, including UBC, due to altered activation/detoxification rates of carcinogens.[3] Almost two thirds of all the UBC cases are shown to be associated with smoking. PAHs, tobacco-specific N-nitrosamines and aromatic amines are the

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Table 1. Clinical and demographic data of the study population.

Variables		Controls	Patients	*P* Value
Age ± SD (years)		64.2 ± 12.82	66.4 ± 13.21	.059
Male, no (%)		200 (80)	198 (79.2)	.92
Female, no (%)		50 (20)	52 (20.8)	
BMI ± SD (kg/m^2)		27.49 ± 4.22	28.3 ± 3.46	.019
Non-Smoker, no (%)		169 (67.6)	49 (19.6)	< .001
Smoker, no (%)		81 (32.4)	201 (80.4)	
Tumor grade, (%)	Low	-	61.2	-
	High	-	38.8	-
Tumor stage, (%)	Superficial	-	62.8	-
	Invasive	-	37.2	-

Abbreviations: BMI, body mass index; SD, standard deviations.

significant contributors to the carcinogenic effects of tobacco smoke via DNA adducts or formation of reactive oxygen species (ROS) leading to oxidative damage and ultimately DNA mutations.[11-13] The balance between the activation and detoxification of these carcinogens by Phase I and Phase II enzymes affect the amount of accumulating DNA damage. Aldoketoreductases (AKRs) are Phase I enzymes that belong to a superfamily of NAD(P)H-linked oxidoreductases and catalyze the conversion of carbonyl group-containing xenobiotics to alcohols for conjugation reactions.[14] Since they have been shown to activate polycyclic aromatic hydrocarbons (PAHs) and nitrosamines in exhaust and cigarette smoke, AKR family members are considered important for smoking-related cancers.[15] The human AKR genes (AKR 1, 6, 7) are highly polymorphic, where variant genotypes may cause alterations in respective protein functions, and act as modifiers of UBC risk. AKR1C3 on chromosome 10p15-p14 is known to act on various subtrates including hormones and PAHs.[12] In addition to that, in vitro studies have shown significant induction of AKR1C3 upon exposure to cigarette smoke condensate, diesel exhaust and PAHs.[13-16]

On this background we hypothesized that genetic variations in the AKR1C3 gene may contribute to UBC risk. AKR polymorphisms have been previously investigated in a total of six studies for their possible involvement in various cancer susceptibilities,[17-22] however, there is only one study investigating the involvement of AKR1C3 polymorphisms in bladder cancer.[17] In this work we aimed to investigate the association of two single nucleotide polymorphisms (SNPs), rs12529 (c.15C > G; p.His5Gln) and rs1937920 (12259 bp 3' of STP A > G), with the risk of developing UBC. rs12529 results in an amino acid substitution and has previously been shown to be inversely associated with bladder cancer risk.[17] There is no enough data to conclude on the physiological effects of this SNP, however, according to current literature it is possible that homozygous variant genotype may induce AKR1C3 expression to facilitate an efficient bioactivation process. rs1937920, residing in the 3'UTR of the AKR1C3 gene, has also been implicated previously in an association study[17] and has the potential to have regulatory roles in AKR1C3 expression.

MATERIALS AND METHODS

Study Population

Total of 500 Caucasian subjects of Turkish origin were included in the study. The clinical and demographic data of the patients and controls were provided in **Table 1**. Cases include 250 first-time diagnosed and histologically confirmed transitional cell carcinoma patients who had not received previous chemotherapy or radiotherapy. Patient selection was not subjected to age, gender or tumor stage/grade restrictions. Patients who had received previous radiotherapy, chemotherapy or radical cystectomy, patients with previous diagnosis of cancer, metastasized cancer, serum prostate-specific antigen (PSA) > 2.5 ng/mL, and those with bladder tumors secondary to other malignancies were excluded from the study. Clinical diagnosis of UBC was given by expert urologists based on urine cytology, urinary tract imaging and cystoscopic analysis. When abnormal tissue is recognized during cystoscopy, transurethral resection of the bladder tumor (TURBT) was performed. Tumors were classified according to the histologic tumor grading system of the World Health Organization (WHO) guidelines.[23] Pathological staging of the tumors was performed according to the TNM classification of malignant tumors,[24] where pTa and pT1 tumors were grouped as superficial and pT2-pT4 tumors were considered invasive. Tumors were graded as low grade and high grade tumors.

The control group includes age- and gender-matched healthy individuals admitted to the hospitals for routine check-up examination with no present or previous history of cancer. Subjects with any degree of hematuria, urinary symptoms, benign prostate hyperplasia (BPH), history of prostatitis and pre-cancerous lesions were excluded from the study.

The study protocol was approved by Haliç University Human Research Ethics Committee. All subjects have provided a written informed consent prior to their inclusion in the study in accordance with Helsinki Declaration, revised in 2000. Data regarding age, gender,

Table 2. Genotype and allele distributions for rs12529 and rs1937920.

Variables	rs12529				
	CC	CG	GG	C	G
	no (%)	no (%)	no (%)	no (%)	no (%)
Patients	32 (12.8)	200 (80)	18 (7.2)	264 (52.8)	236 (47.2)
Controls	40 (16)	146 (58.4)	64 (25.6)	226 (45.2)	274 (54.8)
P value for HWE	.007152				
	rs1937920				
	AA	AG	GG	A	G
	no (%)	no (%)	no (%)	no (%)	no (%)
Patients	101 (40.4)	121 (48.4)	28 (11.2)	323 (64.6)	177 (35.4)
Controls	120 (48)	115 (46)	15 (6)	355 (71)	145 (29)
P value for HWE	.089977				
r^2 for LD	.435795				

Abbreviations: HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium.

ethnicity, occupation, weight, height, family history of cancer, personal medical history and cigarette consumption were collected upon completion of standardized questionnaires. Family history of bladder cancer was considered positive when first or second degree relatives of the subjects had given bladder cancer diagnosis. None of the subjects included in the study were under occupational exposure to hazardous carcinogens related to bladder cancer. Subjects were classified as smokers (who had smoked more than 100 cigarettes in his lifetime) and non-smokers. Ten mL blood samples were collected from patients in EDTA-containing tubes

Table 3. Associations of rs12529 and rs1937920 genotypes and alleles with bladder cancer risk.

Genotype/Allele	OR		95% CI	*P* Value	*P** Value
	rs12529				
CC	Referent				
CG	1.712		1.027-2.856	.038	.076
GG	0.352		0.175-0.708	.003	.006
CC vs (CG+GG)	1.298		0.785-2.144	.308	.616
(CC+CG) vs GG	4.435		2.540-7.744	< .0001	< .0001
C	Ref.				
G	0.737		0.575-0.946	.016	.033
CAT	0.606	-		.002	-
	rs1937920				
AA	Referent				
AG	1.250		0.865-1.806	.234	.468
GG	2.218		1.123-4.381	.020	.040
AA vs (AG+GG)	1.362		0.956-1.940	.087	.174
(AA+GG) vs GG	0.506		0.263-0.973	.038	.076
A	Referent				
G	1.342		1.028-1.751	.030	.061
CAT	1.425	-		.024	-

Abbreviations: CAT, Cochran -Armitage Trend test; OR, odds ratio; CI, confidence interval.
All P values were adjusted for age, gender, BMI and smoking status.
*P**: *P* values after Bonferroni correction.

Table 4. Associations of rs12529 and rs1937920 genotypes with tumor grade and stage

Genotype/	Grade		OR	95 % CI	*P* Value	*P** Value	Stage		OR	95 % CI	*P* Value	*P** Value
Allele	Low	High					Superficial	Invasive				
rs12529												
CC	11	21	Referent				18	14	Referent			
CG	132	68	0.270	0.123-0.592	.00064	.00192	128	72	0.723	0.654-1.352	.399	1.0
GG	10	8	0.419	0.129-1.366	.145	.436	11	7	0.818	0.252-2.655	.738	1.0
C	154	110	Referent				164	100	Referent			
G	152	84	0.774	0.539-1.111	.164	.492	150	86	0.940	0.654-1.352	.740	1.0
rs1937920												
AA	61	40	Referent				65	39	Referent			
AG	72	49	1.038	.605-1.780	.892	1.0	70	50	1.190	0.695-2.039	.525	1.0
GG	20	8	0.610	.245-1.518	.285	.855	22	8	0.606	0.246-1.493	.273	.819
A	194	129	Referent				200	128	Referent			
G	112	65	.873	.598-1.274	.48052	1.0	114	66	0.905	0.621-1.317	.60086	1.0

Abbreviations: OR, odds ratio; CI, confidence interval.
All *P* values were calculated for allelic and additive model and adjusted for age, gender, BMI and smoking status.
*P**: *P* values after Bonferroni correction.

and stored at 4°C until DNA extraction. The control subjects have provided cheek epithelial cells on cotton swabs as the source of DNA.

Genotyping

DNA was isolated from the whole blood samples of patients using High Pure PCR Template Preparation Kit (Roche, Germany) and from buccal swabs using Gentra Puregene Buccal Cell kit (Qiagen, Maryland, USA). Isolated DNA samples were quantified by spectrophotometry and loaded on 1% agarose gels to confirm their integrity. Both single nucleotide polymorphisms (SNPs) were genotyped using Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) methods. PCR-RFLP assays were designed with Biology Workbench using Primer3 and TCAG modules.[25] rs12529 polymorphic region amplified using the forward and reverse primers, 5'-CCTCCTACATGCCATTGGTT-3' and 5'-CAACCCAATACGGGTTTCAC-3', respectively, and the samples were genotyped by Bts-I digestion of the PCR products. rs1937920 polymorphism was genotyped by TaqI restriction of the PCR products amplified using 5'-AGGCAGGCGAACAGAAACTA-3' and 5'-GAAAAACACTTGCATTCGCA-3' primers. The specificity of the PCR products were analyzed by 2% agarose gel electrophoresis, and the digestion products were separated on 3% agarose gels. Genotypes were

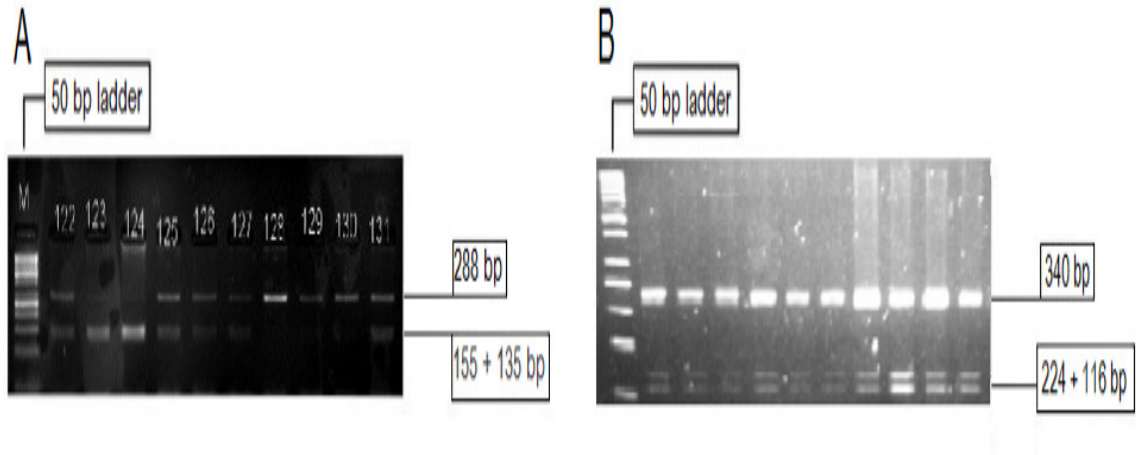

Figure 1. Bts-I (A) and TaqI (B) digestion products run on 3% agarose gels for 15 minutes at 120 volts (50bp ladder, ThermoFisher, California-USA).

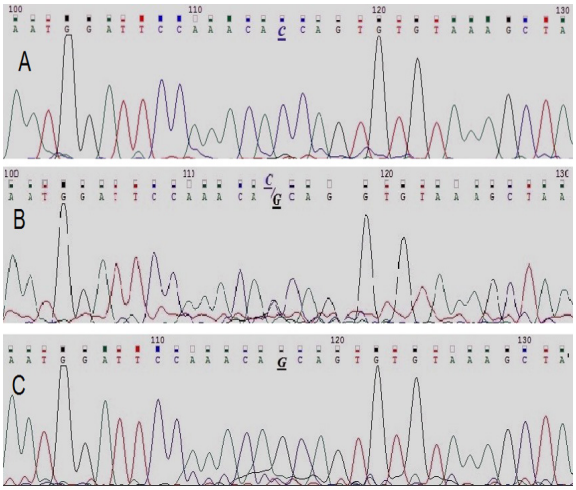

Figure 2. Sanger sequencing results for rs12529. Electropherogram for the wild type homozygous **(A)**, heterozygous **(B)** and homozygous variant **(C)** genotypes.

determined according to the RFLP patterns on the gels (**Figure 1**) and confirmed by Sanger sequencing[26] of 50 randomly chosen samples (**Figure 2**).

Statistical Analysis

The control samples were tested for Hardy-Weinberg equilibrium (HWE) with Chi-square test. The associations between UBC risk and genotypes were examined with allelic, additive, dominant and recessive models using logistic regression. While Cochran-Armitage Trend test was used to obtain a general odds ratio (OR) for both SNPs, it was essentially used for rs12529, since genotype distribution was found to be departed from HWE. The demographic data have shown normal distribution among patients and controls according to the Shapiro-Wilk test results ($P < .05$). Therefore, differences between the continuous variables were assessed using t test. Fisher's exact test was used for the assessment of categorical variables. All the reported P values are two-tailed and adjusted for confounding factors including age, gender, body mass index (BMI) and smoking status. To account for multiple comparisons, Bonferroni correction was applied. Statistical Package for the Social Science (SPSS Inc, Chicago, Illinois, USA) version 21.0 was used for all statistical analyses and $P < .05$ was considered statistically significant. Statistical power of 80% was considered as the lowest acceptable power score. Power calculation was performed according to the method suggested by Kelsey and colleagues.[27] Previously reported genotype frequencies for the Caucasian population were used as hypothetical exposure rates. The sample size for the minimally acceptable power was calculated for rs12529 and rs1937920 as 65 cases and 65 controls with 130 subjects, and 71 cases and 71 controls with 142 subjects, respectively.

RESULTS

The allelic and genotypic distributions for rs12529 and rs1937920 SNPs, together with HWE and linkage disequilibrium (LD) calculations are shown in **Table 2**. According to the results, the two studied SNPs were not in LD ($r^2 = .44$). Both SNPs were shown to be significantly associated with UBC risk (**Table 3**). Our results point out rs12529 as a protective variant ($OR_{trend} = 0.606$; $p_{trend} = .002$) and rs1937920 as a risk variant ($OR_{trend} = 1.425$; $p_{trend} = .024$) for UBC.

For the rs12529 SNP, frequency of the G allele was found to be significantly different between patients and controls ($P^* = .016$). The homozygous variant GG genotype is shown to decrease the risk of UBC (95% CI: .175-.708, OR = 0.352, $P^* = .006$). This effect is also apparent when homozygous wild type and heterozygous genotypes (CC+CG) were tested against the homozygous variant (95% CI: 2.540-7.744, OR = 4.435, $P^* < .0001$). The frequency of the rs1937920 variant allele was also significantly different between patients and controls ($P^* = .03$). The variant genotype GG was identified to be a significant risk factor for UBC (95% CI: 1.123-4.381, OR = 2.218, $P^* = .04$), where the GG genotype raises the odds of developing UBC by 2.2 folds. The associations of the alleles and genotypes with tumor grades and stages are shown in **Table 4**. The heterozygous genotype for rs12529 appears to be significantly associated with tumor grade ($P^* = .002$). However, since the significance decreases in the dominant model, this seems to be an artificial effect due to high heterozygous genotype frequency and could be negated in a study with larger sample size. There was no other significant associations between the tumor grades/stages and the studied polymorphisms.

DISCUSSION

Our results identified the 3' UTR SNP, rs1937920, as a risk variant and the nonsynonymous rs12529 SNP as a protective variant for UBC, thereby provided evidence that AKR1C3 locus is important in the molecular pathogenesis of UBC. The number of studies investigating the association of AKR1C3 polymorphisms with cancer is limited to a few. In three of the six studies prostate cancer samples were analyzed,[20-22] and the rest are three individual studies on non-Hodgkin lymphoma, lung cancer and urinary bladder cancer.[17-19]

rs1937920 polymorphism has been identified in a Spanish Bladder Cancer Study using Golden Gate Assay[17] and minor allele frequency was reported to be 0.29 in the control population. To the best of our knowledge this is the first study investigating the rs1937920 SNP

in relation to bladder cancer. According to our results, homozygous variant genotype carriers of the Turkish population are at 2.2 folds more risk of developing UBC. It can be speculated that this SNP may result in decreased AKR1C3 expression and/or activity, leading to inefficient bioactivation process. Further association studies with large and different ethnic groups and tissue/in vitro expression studies are required in order to clarify the role of this polymorphism in UBC.

We have identified the nonsynonymous rs12529 SNP to be inversely associated with UBC risk. To the best of our knowledge, our results constitute the second contribution to the literature in terms of the involvement of rs12529 in UBC. Here we report the association of the homozygous variant GG genotype with decreased risk for UBC (OR_{GG} = 0.352; 95% CI: 0.175 - 0.708; *P** = .006). Our results corroborated the findings of Figuroea and colleagues[17] who previously reported an inverse association of UBC with this polymorphism in the Spanish population (95 % CI : 0.52-1.18, OR = 0.78, *P* = .04). These two consistent data in the literature support the idea that AKR1C3 c.15 C > G polymorphism may contribute significantly to UBC pathogenesis via induction of AKR1C3 expression to facilitate an efficient bioactivation process. However, this hypothesis should be confirmed with AKR1C3 expression analyses in UBC patients. Interestingly, the heterozygous genotype for rs12529 shows a trend towards significance (*P** = .076) in terms of its association with increased UBC risk in our study. However, since the significance of this effect decreases in dominant model, this seems to be an artificial effect as a result of high heterozygous genotype frequency and could be negated in a study with larger sample size. Regarding the associations of the alleles and genotypes with tumor characteristics, the only significant association was between the heterozygous genotype for rs12529 and tumor grade (*P** = .00192). However, this is possibly due to high representation of the heterozygotes. We could not detect any other associations between the tumor characteristics and the studied genotypes.

The association of rs12529 was investigated previously in Chinese lung cancer patients by Lan et al.[18] who reported two folds increased risk for the homozygous variant genotype (GG). In another study by Lan and colleagues, no association between AKR1C3 variants and non-Hodgkin lymphoma was reported.[19] Cunningham and colleagues on the other hand, investigated AKR1C3 variants in sporadic and familial prostate cancer, but could not find an association between rs12529 and prostate cancer risk.[20] Two other studies on prostate cancer have revealed significant association of AKR1C3 variants and prostate cancer risk when gene interactions were in taken into consideration.[21,22]

While the discrepancy on the role of rs12529 SNP can be attributed to differences in the pathogeneses of lung and urinary bladder cancers, a unique gene-gene or gene-environment effects specific to Chinese population is more likely. This is also supported by the fact that rs12529 genotype distributions in Asian populations are significantly different than that of Caucasians such that Asians have the frequency of 0.770 for the G allele, which was designated as the minor allele for other populations.

CONCLUSIONS

In conclusion, our results suggest that AKR1C3 rs12529 and rs1937920 variants have significant contributions to the risk of developing UBC. Because of the case–control design of this study, population stratification and admixture effects may increase the type I error rate of association; therefore, the results presented here should be regarded as preliminary and might represent a first step of future larger studies aiming to better elucidate the role of AKR1C3 polymorphisms in the susceptibility to bladder cancer.

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CONFLICT OF INTEREST

None declared.

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