Familial Cancer (2017) 16:577–594
DOI 10.1007/s10689-017-9991-z

ORIGINAL ARTICLE

Identification of novel potential genetic predictors of urothelial bladder carcinoma susceptibility in Pakistani population

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Abstract Urothelial bladder carcinoma (UBC) is the most common among urinary bladder neoplasms. We carried out a preliminary study to determine the genetic etiology of UBC in Pakistani population, for this 25 sequence variants from 17 candidate genes were studied in 400 individuals by using polymerase chain reaction-based techniques. Multivariate logistic regression analysis was performed for association analysis of the overall data as well as the data stratified by smoking status, tumor grade and tumor stage. Variants of *GSTM1, IGFBP3, LEPR* and *ACE* were found to be associated with altered UBC risk in the overall comparison. *CYP1B1* and *CDKN1A* variants displayed a risk modulation among smokers; *IGFBP3* and *LEPR* variants among nonsmokers while *GSTM1* polymorphism exhibited association with both. *GSTM1* and *LEPR* variants conferred an altered susceptibility to low grade UBC; *GSTT1, IGFBP3* and *PPARG* variants to high grade UBC while *ACE* polymorphism to both grades. *GSTM1* and *LEPR* variants exhibited risk modulation for non-muscle-invasive bladder cancer (NMIBC); *GSTT1* and *PPARG* variants for muscle-invasive bladder cancer (MIBC), and *ACE* variant for NMIBC as well as MIBC. In general, the susceptibility markers were common for low grade and NMIBC; and distinct from those for high grade and MIBC indicating the distinct pathologies of both groups. In brief, our results conform to reports of previously associated variants in addition to identifying novel potential genetic predictors of UBC susceptibility.

Keywords Genetic predictors · Urothelial bladder carcinoma · Genetic association study · Genetic polymorphisms · Pakistan · Cancer

Introduction

Urinary bladder cancer is the ninth most frequent neoplasm around the globe, affecting approximately 2.7 million people and in 2002 caused 145,000 mortality worldwide [45]. Due

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Electronic supplementary material The online version of this article (doi:10.1007/s10689-017-9991-z) contains supplementary material, which is available to authorized users.

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to the lack of a national cancer registry in Pakistan, a true age-standardized incidence rate is difficult to ascertain, however, Rafique and Javed [47] reported urinary bladder cancer to be the most common urological cancer in both genders in Pakistan.

Bladder cancer has a multifactorial etiology in which extrinsic risk factors such as cigarette smoking and occupational exposure to carcinogens [55]; as well as genetic variations contribute towards modulation of the incidence risk [23]. These environmental and genetic factors also work interactively, complementing or counteracting each other in modulating the risk of the disease [55].

Population-based case control genetic association studies have also been performed in different parts of the world to identify the disease susceptible loci [23]. Some of these genetic factors alter protein activity such as those spanning the coding sequence of gene, e.g. rs1042522 of *TP53* [57] and rs1695 of *GSTP1* [28]; some affect the transcriptional regulation of target genes, e.g. rs9642880 affects *MYC* gene expression [61] and rs2854744 of *IGFBP3* [17]; while some polymorphisms are responsible for the loss of enzymatic activity, e.g. null gene polymorphisms of *GSTT1* and *GSTM1* genes [48].

The present investigation was carried out on the most common type of urinary bladder neoplasms, i.e. urothelial bladder carcinoma (UBC) patients and controls of Pakistani origin. To the best of the authors' knowledge, this is the first extensive report of UBC genetic association study conducted on this population. In this connection, different polymorphic sites were selected based on their biological plausibility and/or information from previously reported studies from other populations.

Since carcinogenesis is a complex and multi-step process that progressively develops from alterations in different cellular pathways [6], therefore genetic variants from some of these pathways were selected in this analysis, including carcinogen metabolism and antioxidant pathways, cell cycle regulation pathway, growth regulation pathway, angiogenesis pathway, folate metabolism pathway, cell signaling pathway, nitric oxide metabolism pathway, inflammatory cytokine and transcription regulation pathway. Previously, we analyzed the association of selected genome-wide association study (GWAS) variants in our population and the results of three common variants from 8q24 region (rs9642880, rs6983267 and rs2294008) have already been published elsewhere [4]. Here the results of 25 common genetic variants (Table 1) are presented.

Materials and methods

Selection criteria for cases and controls

In the present study, unrelated individuals suffering from UBC were recruited from different medical centers in northern Punjab. The cases (N = 200) went through a thorough examination including cystoscopy and *trans*-urethral resection of bladder tumor. The resected tissue specimens were histopathologically analyzed to determine the type, stage and grade of the tumor in order to classify different sub-groups for comparison. On the basis of tumor stage, two sub-groups were categorized: non-muscle invasive bladder cancer (NMIBC) and muscle-invasive and advanced stages into the group muscle-invasive bladder cancer (MIBC). While the samples based upon tumor grades were divided into two groups as defined by WHO 1973 scheme: papilloma, grade-I and grade-II were combined in the low grade group, while grade-III tumors were placed in the high grade group. Cases of non-Pakistani origin and bladder cancer types other than urothelial were not included in the study in addition to those with a previous history of tumor in an organ other than bladder as well as metastasized cancer.

Age, ethnicity and gender-matched healthy controls free from any malignancy were sampled from the general population.

Blood sampling and genomic DNA extraction

The present investigation conformed to the tenets of 1964 Helsinki Declaration and IRB protocols. The study was approved by the "Ethics Review Board" of the Department of Biosciences of the COMSATS Institute of Information Technology, Islamabad, Pakistan. Genomic DNA was extracted by conventional organic method [54] from peripheral leucocytes. For this study prior informed written consent was obtained from all the study participants.

Genotyping

PCR-based protocols were used for the genotyping of selected polymorphisms (Online Resource 1).

Quality control

In order to authenticate the genotyping methods, 10% selected samples representing all the genotypes were confirmed by Sanger DNA sequencing, in addition another 10% were validated by randomly replicating the PCR-based genotyping. Both the validation approaches gave

Table 1 Details of genetic variants analyzed for association with urothelial bladder carcinoma

Pathway/major role	Gene	Chromosomal location	Variant	Variant type	Change	References
Carcinogen metabolism and antioxidant pathway	*CYP1B1*	2p22.2	rs2567206	Promoter region SNP	c.-2805C>T	Han et al. [27]
	GSTT1	22q11.23	–	Null gene variant	–	Rebbeck [48]
	GSTM1	1p13.3	–	Null gene variant	–	Rebbeck [48]
	GSTP1	11q13.2	rs1695	Nonsynonymous SNP	p.Ile105Val	Harries et al. [28]
	PON1	7q21.3	rs854560	Nonsynonymous SNP	p.Leu55Met	Fang et al. [20]
			rs662	Nonsynonymous SNP	p.Gln192Arg	Fang et al. [20]
Cell cycle regulation pathway	*TP53*	17p13.1	rs1042522	Nonsynonymous SNP	p.Pro72Arg	Su et al. [57]
	CDKN1A	6p21.2	rs1801270	Nonsynonymous SNP	p.Ser31Arg	Su et al.[57]
Growth regulation pathway	*IGFBP3*	7p12.3	rs2854744	5′UTR SNP	c.-202A>C	Deal et al. [17]
	LEP	7q32.1	rs7799039	Promoter region SNP	c.-2548G>A	Hoffstedt et al. [30]
	LEPR	1p31.3	rs1137101	Nonsynonymous SNP	p.Gln223Arg	Quinton et al. [46]
Angiogenesis	*VEGFA*	6p21.1	rs2010963	Promoter region SNP	c.-634G>C	Lu et al. [37]
	ACE	17q23.3	rs4646994	*Alu* repeat insertion/deletion polymorphism	–	Zhang et al. [65]
Folate metabolism pathway	*MTHFR*	1p36.22	rs1801133	Nonsynonymous SNP	p.Ala222Val	Safarinejad et al. [52]
			rs1801131	Nonsynonymous SNP	p.Glu429Ala	Safarinejad et al. [52]
			rs2274976	Nonsynonymous SNP	p.Arg594Gln	Safarinejad et al. [52]
Cell signaling pathway	*CAV1*	7q31.2	rs3807987	Intronic SNP	c.14713G>A	Bau et al. [8]
			rs7804372	Intronic SNP	c.29107T>A	Bau et al. [8]
			rs3757733	Intronic SNP	c.28608T>A	Bau et al. [8]
			rs3807992	Intronic SNP	c.32124G>A	Bau et al. [8]
			rs1997623	Intronic SNP	c.239C>A	Bau et al. [8]
			rs12672038	Intronic SNP	c.21985G>A	Bau et al. [8]
Nitric oxide metabolism	*NOS3*	7q36.1	–	VNTR polymorphism	–	Ayub et al. [5]
Inflammatory cytokine	*TNFA*	6p21.33	rs1800629	Promoter region SNP	c.-308G>A	Marsh et al. [40]
Nuclear receptor, transcription regulation	*PPARG*	3p25.2	rs1801282	Nonsynonymous SNP	p.Pro12Ala	Deeb et al. [18]

SNP single nucleotide polymorphism, *5′UTR* 5′ untranslated region, *VNTR* variable number tandem repeat

100% concordance to the genotypes observed in the first attempt.

Data analysis

Statistical procedures used for data analysis included Student's *t* test for the comparison of average age of cases and controls. Hardy–Weinberg Equilibrium (HWE) of genotype frequencies among controls was tested by a goodness-of-fit Chi square (χ^2) test. Odds ratio (OR) and 95% confidence interval (CI) were computed by multivariate logistic regression analysis after controlling for age, gender and smoking to determine the association of the variants.

In-silico analysis

The effects of associated nonsynonymous polymorphisms on the three-dimensional structure of respective proteins were predicted through an online tool, Have Your Protein Explained (HOPE; http://www.cmbi.ru.nl/hope/input/).

Results

In the present investigation, 200 UBC and an equal number of control samples were collected. Male:female ratio of the cases was ~3:1 (78.5 and 21.5%), controls were selected in the same proportion (77.5% males and 22.5% females) to match the gender distribution and average age (UBC = 55.5 SD ±13.24 years; controls = 54.3, SD ±9.9 years; t = 1.03, p = 0.31). There were 92 (46%) cigarette smokers among cases and 72 (36%) among controls, while non-smokers were 108 (54%) in cases and 128 (64%) in the controls. Smoking was found to increase UBC susceptibility in men (OR 1.67, 95% CI 1.04–2.7) but not in women.

Table 2 Overall and smoking status-based comparison of the genotype and allele frequencies among urothelial bladder carcinoma cases and healthy controls

Gene/variant	Geno-type/allele	Cases N = 200	Controls N = 200	OR (95% CI)	Smokers			Non-smokers		
					Cases N = 92	Controls N = 72	OR (95% CI)	Cases N = 108	Controls N = 128	OR (95% CI)
CYP1B1	CC	0.495	0.425	Ref.	0.467	0.458	Ref.	0.519	0.406	Ref.
rs2567206	CT	0.40	0.49	0.7 (0.5–1.1)	0.38	0.486	0.8 (0.4–1.4)	0.417	0.492	0.7 (0.4–1.1)
	TT	0.105	0.085	1.1 (0.5–2.2)	0.152	0.056	2.9 (0.9–10)	0.065	0.102	0.5 (0.2–1.3)
	C	0.695	0.67		0.658	0.70		0.727	0.652	
	T	0.305	0.33	0.9 (0.7–1.2)	0.342	0.30	1.3 (0.8–2)	0.273	0.348	0.7 (0.5–1.02)
	RM						**3.5 (1.05–11.5)**			
GSTT1	T1	0.83	0.87		0.86	0.89		0.806	0.86	
	T0	0.17	0.13	1.4 (0.8–2.5)	0.14	0.11	1.3 (0.5–3.4)	0.194	0.14	1.4 (0.7–2.9)
GSTM1	M1	0.585	0.715		0.64	0.78		0.537	0.68	
	M0	0.415	0.285	**1.9 (1.3–2.9)**	0.36	0.22	**1.96 (1.1–3.96)**	0.463	0.32	**1.9 (1.1–3.2)**
GSTP1	AA	0.525	0.56	Ref.	0.489	0.514	Ref.	0.556	0.586	Ref.
rs1695	GA	0.385	0.37	1.1 (0.7–1.7)	0.438	0.458	1 (0.5–1.93)	0.343	0.32	1.2 (0.7–2)
	GG	0.09	0.07	1.4 (0.7–3)	0.076	0.028	2.9 (0.6–15)	0.102	0.094	1.1 (0.5–2.8)
	A	0.72	0.745		0.707	0.743		0.727	0.746	
	G	0.28	0. 255	1.2 (0.8–1.6)	0.293	0.257	1.2 (0.7–2)	0.273	0.254	1.1 (0.8–1.6)
PON1	AA	0.57	0.585	Ref.	0.619	0.556	Ref.	0.528	0.602	Ref.
rs854560	AT	0.39	0.365	1.1 (0.7–1.7)	0.359	0.361	0.9 (0.5–1.7)	0.417	0.367	1.3 (0.8–2.2)
	TT	0.04	0.05	0.8 (0.3–2.1)	0.022	0.038	0.2 (0.04–1.2)	0.056	0.031	1.9 (0.5–7.3)
	A	0.765	0.77		0.80	0.736		0.736	0.785	
	T	0.235	0.23	1.01 (0.7–1.4)	0.20	0.264	0.7 (0.4–1.2)	0.264	0.215	1.3 (0.9–2.1)
rs662	AA	0.49	0.46	Ref.	0.446	0.458	Ref.	0.528	0.461	Ref.
	GA	0.40	0.45	0.8 (0.5–1.3)	0.435	0.431	1.05 (0.5–2)	0.37	0.461	0.7 (0.4–1.2)
	GG	0.11	0.09	1.1 (0.6–2.2)	0.12	0.111	1.2 (0.4–3.2)	0.102	0.078	1.1 (0.4–2.8)
	A	0.69	0.685		0.663	0.674		0.713	0.69	
	G	0.31	0.315	0.96 (0.7–1.3)	0.337	0.326	1.1 (0.7–1.8)	0.287	0.31	0.9 (0.6–1.3)
TP53	CC	0.265	0.29	Ref.	0.30	0.347	Ref.	0.231	0.258	Ref.
rs1042522	CG	0.435	0.455	1.2 (0.–1.9)	0.37	0.417	1.1 (0.5–2.2)	0.491	0.477	1.2 (0.6–2.3)
	GG	0.30	0.255	1.3 (0.8–2.3)	0.33	0.236	1.6 (0.7–3.6)	0.278	0.266	1.2 (0.6–2.4)
	C	0.48	0.52		0.49	0.556		0.477	0.496	
	G	0.52	0.48	1.2 (0.9–1.5)	0.51	0.444	1.3 (0.9–1.9)	0.523	0.504	1.1 (0.8–1.5)
CDKN1A	CC	0.86	0.83	Ref.	0.89	0.764	Ref.	0.833	0.867	Ref.

Table 2 (continued)

Gene/variant	Genotype/allele	Cases N = 200	Controls N = 200	OR (95% CI)	Smokers			Non-smokers		
					Cases N = 92	Controls N = 72	OR (95% CI)	Cases N = 108	Controls N = 128	OR (95% CI)
rs1801270	CA	0.125	0.15	0.8 (0.5–1.5)	0.11	0.208	0.5 (0.2–1.1)	0.139	0.117	1.3 (0.6–2.8)
	AA	0.015	0.02	0.8 (0.2–3.5)	0.00	0.028	NA	0.028	0.016	1.9 (0.3–11.8)
	C	0.92	0.905		0.946	0.868		0.903	0.926	
	A	0.08	0.095	0.8 (0.5–1.3)	0.054	0.132	**0.4 (0.2–0.7)**	0.097	0.074	1.3 (0.7–2.4)
IGFBP3	CC	0.31	0.21	Ref.	0.28	0.25	Ref.	0.333	0.188	Ref.
rs2854744	CA	0.51	0.57	**0.6 (0.4–0.97)**	0.49	0.556	0.8 (0.4–1.6)	0.528	0.578	**0.5 (0.3–0.9)**
	AA	0.18	0.22	**0.5 (0.3–0.97)**	0.23	0.194	1.01 (0.4–2.5)	0.139	0.234	**0.3 (0.14–0.7)**
	C	0.565	0.495		0.527	0.528		0.597	0.477	
	A	0.435	0.505	0.8 (0.6–1.01)	0.473	0.472	1 (0.6–1.6)	0.403	0.523	**0.6 (0.4–0.9)**
LEP	GG	0.18	0.195	Ref.	0.207	0.139	Ref.	0.157	0.227	Ref.
rs7799039	GA	0.515	0.50	1.1 (0.7–1.9)	0.50	0.444	0.8 (0.3–1.9)	0.528	0.531	1.4 (0.7–2.8)
	AA	0.305	0.305	1.04 (0.6–1.9)	0.293	0.417	0.5 (0.2–1.2)	0.315	0.242	1.9 (0.9–4.02)
	G	0.44	0.445		0.457	0.36		0.421	0.492	
	A	0.56	0.555	1.01 (0.8–1.4)	0.543	0.64	0.7 (0.4–1.1)	0.579	0.508	1.4 (0.9–1.99)
LEPR	AA	0.252	0.335	Ref.	0.293	0.306	Ref.	0.222	0.35	Ref.
rs1137101	AG	0.48	0.485	1.3 (0.8–2.04)	0.478	0.514	0.94 (0.5–1.9)	0.481	0.47	1.6 (0.9–3)
	GG	0.265	0.18	**1.9 (1.1–3.4)**	0.228	0.18	1.3 (0.5–3.1)	0.296	0.18	**2.6 (1.2–5.4)**
	A	0.495	0.58		0.533	0.56		0.463	0.586	
	G	0.505	0.42	**1.4 (1.04–1.8)**	0.467	0.44	1.15 (0.7–1.8)	0.537	0.414	**1.6 (1.1–2.3)**
VEGFA	GG	0.51	0.51	Ref.	0.50	0.542	Ref.	0.52	0.492	Ref.
rs2010963	CG	0.37	0.38	0.99 (0.7–1.5)	0.36	0.319	1.2 (0.6–2.4)	0.38	0.414	0.9 (0.5–1.5)
	CC	0.12	0.11	1.03 (0.5–2)	0.14	0.139	1.1 (0.4–2.7)	0.10	0.094	1.02 (0.4–2.5)
	G	0.695	0.70		0.68	0.70		0.71	0.70	
	C	0.305	0.30	1.01 (0.8–1.3)	0.32	0.30	1.1 (0.7–1.8)	0.29	0.30	0.96 (0.7–1.4)
ACE	II	0.32	0.325	Ref.	0.304	0.389	Ref.	0.333	0.29	Ref.
rs4646994	ID	0.395	0.50	0.8 (0.5–1.3)	0.402	0.458	1.1 (0.6–2.2)	0.389	0.52	0.6 (0.4–1.2)
	DD	0.285	0.175	1.7 (0.97–3)	0.293	0.153	2.4 (0.99–5.8)	0.278	0.19	1.3 (0.6–2.7)
	I	0.52	0.575		0.505	0.618		0.528	0.55	
	D	0.48	0.425	1.2 (0.95–1.6)	0.495	0.382	1.5 (0.98–2.3)	0.472	0.45	1.1 (0.8–1.6)
	RM			**1.9 (1.17–3.1)**			2.3 (1–5.44)			1.67 (0.9–3.2)
MTHFR	CC	0.685	0.65	Ref.	0.75	0.708	Ref.	0.63	0.617	Ref.
rs1801133	CT	0.29	0.31	0.9 (0.6–1.4)	0.228	0.264	0.8 (0.4–1.7)	0.343	0.336	0.97 (0.6–1.7)
	TT	0.025	0.04	0.6 (0.2–1.9)	0.022	0.028	0.7 (0.1–5.3)	0.028	0.047	0.5 (0.1–2.3)
	C	0.83	0.805		0.864	0.84		0.80	0.785	
	T	0.17	0.195	0.9 (0.6–1.2)	0.136	0.16	0.8 (0.5–1.5)	0.20	0.215	0.9 (0.6–1.4)

Table 2 (continued)

Gene/variant	Geno-type/allele	Cases N = 200	Controls N = 200	OR (95% CI)	Smokers			Non-smokers		
					Cases N = 92	Controls N = 72	OR (95% CI)	Cases N = 108	Controls N = 128	OR (95% CI)
rs1801131	AA	0.295	0.365	Ref.	0.304	0.319	Ref.	0.287	0.39	Ref.
	AC	0.62	0.545	1.4 (0.9–2.2)	0.576	0.556	1.1 (0.6–2.2)	0.657	0.54	1.7 (0.97–3)
	CC	0.085	0.09	1.1 (0.5–2.3)	0.12	0.125	0.99 (0.4–2.8)	0.056	0.07	1.1 (0.4–3.3)
	A	0.605	0.64		0.592	0.597		0.616	0.66	
	C	0.395	0.36	1.2 (0.8–1.6)	0.408	0.403	1.05 (0.6–1.7)	0.384	0.34	1.3 (0.8–2.1)
rs2274976	GG	0.81	0.815	Ref.	0.837	0.736	Ref.	0.787	0.859	Ref.
	GA	0.17	0.175	0.96 (0.6–1.6)	0.141	0.264	0.5 (0.2–1.02)	0.194	0.125	1.8 (0.9–3.7)
	AA	0.02	0.01	1.96 (0.4–11)	0.022	0.00	NA	0.019	0.016	1.1 (0.1–7.9)
	G	0.895	0.90		0.908	0.868		0.884	0.922	
	A	0.105	0.10	1.1 (0.7–1.7)	0.092	0.132	0.7 (0.3–1.4)	0.116	0.078	1.5 (0.8–2.7)
CAV1	GG	0.80	0.835	Ref.	0.783	0.778	Ref.	0.815	0.867	Ref.
rs3807987	GA	0.175	0.15	1.2 (0.7–2.2)	0.197	0.208	0.9 (0.4–2)	0.157	0.117	1.4 (0.7–3)
	AA	0.025	0.015	1.7 (0.4–7.1)	0.022	0.014	1.4 (0.1–6.1)	0.028	0.016	1.8 (0.3–11)
	G	0.89	0.91		0.88	0.882		0.894	0.926	
	A	0.11	0.09	1.2 (0.8–1.9)	0.12	0.118	0.97 (0.5–1.9)	0.106	0.074	1.4 (0.8–2.5)
rs7804372	TT	0.57	0.58	Ref.	0.533	0.542	Ref.	0.602	0.602	Ref.
	AT	0.36	0.345	1.02 (0.7–1.6)	0.38	0.417	0.94 (0.5–1.8)	0.343	0.305	1.1 (0.6–1.9)
	AA	0.07	0.075	0.94 (0.4–2.1)	0.087	0.042	2.04 (0.5–8.2)	0.056	0.094	0.59 (0.2–1.7)
	T	0.75	0.75		0.723	0.75		0.773	0.754	
	A	0.25	0.25	0.99 (0.7–1.4)	0.277	0.25	1.4 (0.7–1.9)	0.227	0.246	0.9 (0.6–1.4)
rs3757733	TT	0.605	0.635	Ref.	0.565	0.639	Ref.	0.639	0.633	Ref.
	AT	0.385	0.355	1.1 (0.7–1.7)	0.424	0.347	1.4 (0.7–2.6)	0.352	0.359	0.96 (0.6–1.7)
	AA	0.01	0.01	0.97 (0.1–7.1)	0.011	0.014	0.9 (0.1–14.3)	0.009	0.008	1.1 (0.07–18)
	T	0.80	0.81		0.777	0.812		0.815	0.81	
	A	0.20	0.19	1.1 (0.8–1.6)	0.223	0.188	1.3 (0.8–2.5)	0.185	0.19	0.97 (0.6–1.6)
rs3807992	GG	0.59	0.58	Ref.	0.533	0.556	Ref.	0.639	0.594	Ref.
	AG	0.31	0.345	0.8 (0.6–1.3)	0.348	0.417	0.9 (0.5–1.7)	0.278	0.305	0.8 (0.5–1.5)
	AA	0.10	0.075	1.3 (0.6–2.7)	0.12	0.028	4.4 (0.9–21.2)	0.083	0.102	0.7 (0.3–1.9)
	G	0.745	0.75		0.707	0.764		0.778	0.746	
	A	0.255	0.25	1.01 (0.8–1.4)	0.293	0.236	1.3 (0.8–2.2)	0.222	0.254	0.9 (0.6–1.3)
rs1997623	CC	0.69	0.72	Ref.	0.663	0.708	Ref.	0.713	0.727	Ref.
	AC	0.28	0.27	1.07 (0.7–1.7)	0.293	0.292	1.06 (0.5–2.1)	0.269	0.259	1.1 (0.6–2)
	AA	0.03	0.01	3.4 (0.7–17.4)	0.043	0.00	NA	0.019	0.017	1.3 (0.2–9.7)
	C	0.83	0.855		0.81	0.854		0.847	0.855	
	A	0.17	0.145	1.2 (0.8–1.8)	0.19	0.146	1.4 (0.8–2.9)	0.153	0.145	1.1 (0.7–1.9)

Table 2 (continued)

Gene/variant	Geno-type/allele	Cases N = 200	Controls N = 200	OR (95% CI)	Smokers			Non-smokers		
					Cases N = 92	Controls N = 72	OR (95% CI)	Cases N = 108	Controls N = 128	OR (95% CI)
rs12672038	GG	0.815	0.805	Ref.	0.772	0.75	Ref.	0.852	0.835	Ref.
	GA	0.16	0.17	0.9 (0.5–1.5)	0.196	0.236	0.8 (0.4–1.7)	0.129	0.133	0.98 (0.5–2.1)
	AA	0.025	0.025	0.9 (0.2–3.2)	0.033	0.014	2.1 (0.2–20.5)	0.019	0.031	0.5 (0.9–2.9)
	G	0.895	0.89		0.87	0.868		0.917	0.902	
	A	0.105	0.11	0.9 (0.6–1.4)	0.13	0.132	0.95 (0.5–1.8)	0.083	0.098	0.9 (0.5–1.5)
NOS3	bb	0.72	0.76	Ref.	0.728	0.736	Ref.	0.713	0.773	Ref.
	ba	0.245	0.205	1.3 (0.8–2)	0.228	0.208	1.1 (0.5–2.4)	0.259	0.203	1.4 (0.8–2.6)
	aa	0.035	0.035	0.96 (0.3–2.9)	0.043	0.056	0.8 (0.2–3.2)	0.028	0.023	1.3 (0.3–6.6)
	b	0.84	0.86		0.84	0.84		0.84	0.875	
	a	0.16	0.14	1.1 (0.8–1.7)	0.16	0.16	0.99 (0.6–1.7)	0.16	0.125	1.3 (0.8–2.2)
TNFA	GG	0.86	0.84	Ref.	0.89	0.847	Ref.	0.833	0.836	Ref.
rs1800629	GA	0.13	0.15	0.9 (0.5–1.6)	0.11	0.139	0.8 (0.3–1.96)	0.148	0.156	0.96 (0.5–2)
	AA	0.01	0.01	1.02 (0.1–7.4)	0.00	0.014	NA	0.019	0.008	2.2 (0.2–25.3)
	G	0.925	0.915		0.946	0.917		0.907	0.914	
	A	0.075	0.085	0.9 (0.6–1.5)	0.054	0.083	0.6 (0.3–1.5)	0.093	0.086	1.1 (0.6–2.02)
PPARG	CC	0.755	0.77	Ref.	0.728	0.792	Ref.	0.778	0.758	Ref.
rs1801282	CG	0.205	0.215	0.94 (0.6–1.5)	0.228	0.208	1.2 (0.6–2.5)	0.185	0.219	0.8 (0.4–1.5)
	GG	0.04	0.015	2.8 (0.7–11)	0.044	0.00	NA	0.037	0.023	1.5 (0.3–7)
	C	0.86	0.88		0.842	0.896		0.87	0.87	
	G	0.14	0.12	1.2 (0.8–1.7)	0.158	0.104	1.6 (0.8–3.04)	0.13	0.13	0.95 (0.6–1.6)

Statistically significant values ($p \leq 0.05$) are presented in bold

RM recessive model, *OR (95% CI)* odds ratio (95% confidence interval) adjusted for age, gender and smoking in the overall comparison while for age and gender in smoking-based comparison, *Ref.* reference genotype, *NA* not applicable

Overall analysis

Only few polymorphisms were found to modulate UBC susceptibility after adjusting for age, gender and smoking (Table 2). An increase in bladder tumor susceptibility was conferred by *GSTM1* null gene variant (M0M0/M1M1 + M1M0 OR 1.9, 95% CI 1.3–2.9); *LEPR* rs1137101 [(GG/AA OR 1.9, 95% CI 1.1–3.4); log-additive model (LAM) OR 1.4, 95% CI 1.04–1.8] and *ACE* rs4646994 (DD/II + ID OR 1.9, 95% CI 1.17–3.1). In addition there was a significant protective effect of *IGFBP3* rs2854744 [(CA/CC OR 0.6, 95% CI 0.4–0.97); (AA/CC OR 0.5, 95% CI 0.3–0.97)] against bladder tumor risk.

Smoking-status based analysis

This analysis was performed by comparing smoker cases with smoker controls and non-smoker cases with non-smoker controls (Table 2). *CYP1B1* rs2567206 was found to be associated with high UBC risk among smokers (TT/CC + CT OR 3.5, 95% CI 1.05–11.5) while *CDKN1A* rs1801270 conferred protection to them (LAM OR 0.4, 95% CI 0.2–0.7). *GSTM1* null gene variant was found to increase the risk among both smokers (M0M0/M1M1 + M1M0 OR 1.96, 95% CI 1.1–3.96) and non-smokers (M0M0/M1M1 + M1M0 OR 1.9, 95% CI 1.1–3.2). In addition, *LEPR* rs1137101 was found to enhance UBC susceptibility among non-smokers [(GG/AA OR 2.6, 95% CI 1.2–5.4); (LAM OR 1.6, 95% CI 1.1–2.3)] while *IGFBP3* rs2854744 was found to play a protective role [(CA/CC OR 0.5, 95% CI 0.3–0.9); (AA/CC OR 0.3, 95% CI 0.14–0.7); (LAM OR 0.6, 95% CI 0.4–0.9)]. All other polymorphisms presented a non-significant relationship with tumor risk with respect to smoking status.

Association with tumor characteristics

With reference to the tumor stage and grade, comparisons were carried out for low grade, high grade, NMIBC and MIBC tumors independently against the control population (Table 3).

PPARG rs1801282 conferred an increased susceptibility towards high grade UBC (GG/CC OR 5.97, 95% CI 1.3–26) and MIBC (GG/CC OR 5.4, 95% CI 1.2–24). *GSTT1* null gene polymorphism was found to be associated with an elevated risk of high grade UBC (T0T0/T1T1 + T1T0 OR 2.2, 95% CI 1.1–4.5) and MIBC (T0T0/T1T1 + T1T0 OR 2.7, 95% CI 1.4–5.4) while that of *GSTM1* with increased susceptibility of low grade cancer (M0M0/M1M1 + M1M0 OR 2.2, 95% CI 1.4–3.5) and NMIBC (M0M0/M1M1 + M1M0 OR 2.2, 95% CI 1.3–3.5). *LEPR* rs1137101 was also found to increase the risk of low grade UBC [(GG/AA OR 2.1, 95% CI 1.14–3.9); (LAM OR 1.4, 95% CI 1.1–2)] and NMIBC [(GG/AA OR 2.1, 95% CI 1.1–3.9); (LAM OR 1.4, 95% CI 1.04–2)]. On the other hand, *IGFBP3* rs2854744 conferred protection against high grade UBC (CA/CC OR 0.5, 95% CI 0.3–0.98).

Under a recessive model (DD/II + ID), *ACE* rs4646994 deletion genotype was found to enhance the risk of low grade UBC (OR 1.8, 95% CI 1.07–3.1); high grade UBC (OR 2.07, 95% CI 1.1–4); NMIBC (OR 1.8, 95% CI 1.05–3.1) and MIBC (OR 1.93, 95% CI 1.02–3.7).

Discussion

Urothelial bladder carcinoma is a multifactorial disorder with diverse environmental and genetic etiologies. In the current work, a genetic association study of urothelial bladder carcinogenesis was conducted on a group of UBC cases and controls from Pakistan.

UBC is a disease with a male predominance, the observed gender ratio (~3:1) in the current study is in line with previously reported frequencies of 2–3:1 in Pakistan and other parts of the world [2, 32]. This male-dominated prevalence perhaps is due to the greater exposure of men to environmental carcinogens than women, in addition to hormonal and reproductive factors in the latter [15].

Among the environmental risk factors, cigarette smoking is conventionally considered as one of the biggest known threats [55] and the disease is often referred to as a smoking-related cancer. In agreement with this, the present study too indicated cigarette smoking as one of the risk factors of bladder tumorigenesis. Prevalence of cigarette smoking is 36% in the general male population of Pakistan [3]; while for women, it is considered a taboo. Consequently, we found smoking to be a significant UBC-predisposing factor among men but not in women, as opposed to Karagas et al. [34] who found the risk to equally contribute towards the disease in both genders. However, had a larger number of female smokers been present in the current study, the result would have been more informative.

Among all the selected genetic variants, only few reached a statistical significance to be associated with altered UBC risk and/or its severity. Three of these risk modulators belonged to carcinogen metabolism and antioxidant pathway. Carcinogen exposure and oxidative stress are among the strongest known risk factors for cancer and the tissue has different types of enzymes to deal with these threats. Cytochrome P450 1B1 (encoded by *CYP1B1*) is a phase-I carcinogen metabolizing enzyme and is involved in the metabolic conversion of several exogenous (e.g., polycyclic aromatic hydrocarbons) and endogenous compounds (e.g., estradiol) into carcinogenic metabolites, which in turn induce carcinogenesis [7]. In the current study, presence of TT genotype of a promoter region polymorphism

Table 3 Comparison of the genotype and allele frequencies among the urothelial bladder carcinoma cases and healthy controls with respect to tumor characteristics

Variation	Geno-type/allele	Controls N = 200	Low grade N = 133	OR (95% CI)	High grade N = 67	OR (95% CI)	NMIBC N = 124	OR (95% CI)	MIBC N = 76	OR (95% CI)
CYP1B1	CC	0.425	0.496	Ref.	0.492	Ref.	0.532	Ref.	0.434	Ref.
rs2567206	CT	0.49	0.406	0.7 (0.4–1.3)	0.388	0.7 (0.4–1.2)	0.387	0.6 (0.4–1)	0.421	0.8 (0.5–1.5)
	TT	0.085	0.098	1 (0.5–2.3)	0.119	1.2 (0.5–3.1)	0.081	0.8 (0.4–2)	0.145	1.5 (0.6–3.6)
	C	0.67	0.699		0.69		0.726		0.645	
	T	0.33	0.301	0.9 (0.6–1.2)	0.31	0.9 (0.6–1.4)	0.274	0.8 (0.5–1.1)	0.355	1.1 (0.7–1.6)
GSTT1	T1	0.87	0.865		0.761		0.89		0.74	
	T0	0.13	0.135	1.1 (0.6–2)	0.239	**2.2 (1.1–4.5)**	0.11	0.8 (0.4–1.6)	0.26	**2.7 (1.4–5.4)**
GSTM1	M1	0.715	0.549		0.657		0.556		0.63	
	M0	0.285	0.451	**2.2 (1.4–3.5)**	0.343	1.5 (0.8–2.8)	0.444	**2.2 (1.3–3.5)**	0.37	1.6 (0.9–2.9)
GSTP1	AA	0.56	0.511	Ref.	0.552	Ref.	0.54	Ref.	0.50	Ref.
rs1695	GA	0.37	0.399	1.1 (0.7–1.8)	0.358	1 (0.5–1.8)	0.355	0.97 (0.6–1.6)	0.434	1.4 (0.8–2.4)
	GG	0.07	0.09	1.5 (0.6–3.4)	0.09	1.5 (0.5–4.1)	0.105	1.6 (0.7–3.7)	0.066	1.2 (0.4–3.5)
	A	0.745	0.71		0.73		0.72		0.72	
	G	0. 255	0.29	1.2 (0.8–1.7)	0.27	1.1 (0.7–1.7)	0.28	1.1 (0.8–1.6)	0.28	1.2 (0.7–1.9)
PON1	AA	0.585	0.564	Ref.	0.582	Ref.	0.532	Ref.	0.632	Ref.
rs854560	AT	0.365	0.399	1.1 (0.7–1.8)	0.373	1.4 (0.6–1.9)	0.435	1.3 (0.8–2.1)	0.316	0.8 (0.5–1.4)
	TT	0.05	0.038	0.7 (0.2–2.1)	0.045	0.9 (0.2–3.4)	0.032	0.6 (0.2–1.9)	0.053	1.1 (0.3–3.7)
	A	0.77	0.76		0.77		0.75		0.79	
	T	0.23	0.24	1 (0.7–1.5)	0.23	0.99 (0.6–1.6)	0.25	1.1 (0.7–1.6)	0.21	0.9 (0.6–1.4)
rs662	AA	0.46	0.444	Ref.	0.582	Ref.	0.484	Ref.	0.50	Ref.
	GA	0.45	0.436	1.03 (0.6–1.6)	0.328	0.6 (0.3–1.04)	0.411	0.9 (0.6–1.4)	0.382	0.8 (0.4–1.4)
	GG	0.09	0.12	1.4 (0.7–3.1)	0.09	0.7 (0.2–1.9)	0.105	1.2 (0.5–2.7)	0.118	1.1 (0.4–2.6)
	A	0.685	0.66		0.75		0.69		0.69	
	G	0.315	0.34	1.1 (0.8–1.6)	0.25	0.7 (0.5–1.1)	0.31	1.01 (0.7–1.4)	0.31	0.92 (0.6–1.4)
TP53	CC	0.29	0.226	Ref.	0.343	Ref.	0.266	Ref.	0.263	Ref.
rs1042522	CG	0.455	0.444	1.3 (0.9–2.3)	0.418	0.9 (0.5–1.7)	0.419	1.1 (0.6–1.9)	0.461	1.2 (0.6–2.4)
	GG	0.255	0.331	1.7 (0.9–3)	0.239	0.9 (0.4–1.8)	0.315	1.4 (0.7–2.5)	0.276	1.2 (0.6–2.6)
	C	0.52	0.45		0.55		0.476		0.49	
	G	0.48	0.55	1.3 (0.9–1.7)	0.45	1.1 (0.8–1.4)	0.524	1.2 (0.9–1.6)	0.51	1.1 (0.8–1.6)
CDKN1A	CC	0.83	0.872	Ref.	0.836	Ref.	0.855	Ref.	0.868	Ref.
rs1801270	CA	0.15	0.12	0.8 (0.4–1.5)	0.134	0.9 (0.5–2.05)	0.137	0.9 (0.5–1.7)	0.105	0.6 (0.3–1.4)
	AA	0.02	0.008	0.4 (0.04–3.3)	0.03	1.4 (0.2–7.7)	0.008	0.4 (0.04–3.8)	0.026	1.1 (0.2–6.3)
	C	0.905	0.93		0.9		0.92		0.92	
	A	0.095	0.07	0.7 (0.4–1.3)	0.1	1.01 (0.5–1.9)	0.08	0.8 (0.5–1.4)	0.08	0.8 (0.4–1.5)
IGFBP3	CC	0.21	0.301	Ref.	0.328	Ref.	0.315	Ref.	0.303	Ref.

Table 3 (continued)

Variation	Geno-type/ allele	Controls N = 200	Low grade N = 133	OR (95% CI)	High grade N = 67	OR (95% CI)	NMIBC N = 124	OR (95% CI)	MIBC N = 76	OR (95% CI)
rs2854744	CA	0.57	0.534	0.7 (0.4–1.1)	0.463	**0.5 (0.3–0.98)**	0.524	0.6 (0.4–1.1)	0.487	0.6 (0.3–1.1)
	AA	0.22	0.165	0.5 (0.3–1.03)	0.209	0.6 (0.3–1.26)	0.16	0.5 (0.2–1.01)	0.21	0.6 (0.3–1.3)
	C	0.495	0.57		0.56		0.577		0.546	
	A	0.505	0.43	0.7 (0.5–1.01)	0.44	0.7 (0.5–1.1)	0.423	0.7 (0.5–1)	0.454	0.8 (0.6–1.2)
LEP	GG	0.195	0.181	Ref.	0.179	Ref.	0.202	Ref.	0.145	Ref.
rs7799039	GA	0.50	0.519	1.1 (0.6–2.04)	0.508	1.07 (0.5–2.3)	0.492	0.94 (0.5–1.7)	0.553	1.4 (0.7–3.1)
	AA	0.305	0.301	1.05 (0.6–2)	0.313	0.97 (0.4–2.3)	0.306	0.9 (0.5–1.7)	0.303	1.2 (0.5–2.8)
	G	0.445	0.44		0.43		0.45		0.42	
	A	0.555	0.56	1.01 (0.7–1.4)	0.57	0.98 (0.7–1.5)	0.55	0.99 (0.7–1.3)	0.58	1.1 (0.7–1.6)
LEPR	AA	0.335	0.271	Ref.	0.224	Ref.	0.258	Ref.	0.25	Ref.
rs1137101	AG	0.485	0.429	1.1 (0.7–1.9)	0.582	1.7 (0.9–3.4)	0.452	1.2 (0.7–2)	0.526	1.4 (0.8–2.7)
	GG	0.18	0.301	**2.1 (1.14–3.9)**	0.194	1.6 (0.7–3.7)	0.29	**2.1 (1.1–3.9)**	0.224	1.7 (0.8–3.6)
	A	0.58	0.48		0.51		0.484		0.51	
	G	0.42	0.52	**1.4 (1.1–2)**	0.49	1.3 (0.9–1.9)	0.516	**1.4 (1.04–2)**	0.49	1.3 (0.9–1.9)
VEGFA	GG	0.51	0.481	Ref.	0.567	Ref.	0.508	Ref.	0.51	Ref.
rs2010963	CG	0.38	0.414	1.17 (0.7–1.9)	0.284	0.7 (0.4–1.3)	0.395	1.09 (0.7–1.8)	0.33	0.9 (0.5–1.6)
	CC	0.11	0.105	0.93 (0.4–2)	0.149	1.2 (0.5–2.8)	0.097	0.8 (0.4–1.8)	0.16	1.4 (0.6–3.2)
	G	0.70	0.69		0.71		0.706		0.68	
	C	0.30	0.31	1.03 (0.7–1.4)	0.29	0.97 (0.6–1.5)	0.294	0.97 (0.7–1.4)	0.32	1.1 (0.8–1.6)
ACE	II	0.325	0.316	Ref.	0.328	Ref.	0.282	Ref.	0.382	Ref.
rs4646994	ID	0.50	0.406	0.84 (0.5–1.4)	0.373	0.8 (0.4–1.5)	0.427	1.02 (0.6–1.7)	0.342	0.6 (0.3–1.1)
	DD	0.175	0.278	1.7 (0.4–3)	0.299	1.8 (0.8–3.7)	0.29	1.9 (1–3.7)	0.276	1.5 (0.7–2.9)
	I	0.575	0.52		0.51		0.496		0.55	
	D	0.425	0.48	1.24 (0.9–1.7)	0.49	1.29 (0.9–1.9)	0.504	1.3 (0.97–1.8)	0.45	1.1 (0.8–1.6)
	RM			**1.8 (1.07–3.1)**		**2.07 (1.1–4)**		**1.8 (1.05–3.1)**		**1.93 (1.02–3.7)**
MTHFR	CC	0.65	0.662	Ref.	0.731	Ref.	0.653	Ref.	0.74	Ref.
rs1801133	CT	0.31	0.301	0.96 (0.6–1.6)	0.269	0.8 (0.4–1.5)	0.306	0.98 (0.6–1.6)	0.26	0.7 (0.4–1.4)
	TT	0.04	0.038	0.93 (0.3–3)	0.00	NA	0.04	1 (0.3–3.2)	0.00	NA
	C	0.805	0.81		0.87		0.806		0.87	
	T	0.195	0.19	0.96 (0.6–1.4)	0.13	0.7 (0.4–1.14)	0.194	0.99 (0.7–1.5)	0.13	0.6 (0.3–1.04)
rs1801131	AA	0.365	0.286	Ref.	0.313	Ref.	0.29	Ref.	0.303	Ref.
	AC	0.545	0.632	1.5 (0.9–2.5)	0.597	1.2 (0.7–2.3)	0.597	1.4 (0.8–2.3)	0.658	1.5 (0.8–2.7)
	CC	0.09	0.083	1.13 (0.5–2.6)	0.09	0.95 (0.3–2.8)	0.113	1.6 (0.7–3.5)	0.039	0.5 (0.1–1.8)
	A	0.64	0.60		0.61		0.59		0.63	
	C	0.36	0.40	1.2 (0.8–1.8)	0.39	1.07 (0.7–1.7)	0.41	1.3 (0.9–1.9)	0.37	1.01 (0.6–1.6)

Table 3 (continued)

Variation	Geno-type/ allele	Controls N = 200	Low grade N = 133	OR (95% CI)	High grade N = 67	OR (95% CI)	NMIBC N = 124	OR (95% CI)	MIBC N = 76	OR (95% CI)
rs2274976	GG	0.815	0.812	Ref.	0.806	Ref.	0.79	Ref.	0.842	Ref.
	GA	0.175	0.173	0.97 (0.5–1.7)	0.164	0.9 (0.4–1.8)	0.202	1.1 (0.6–2.02)	0.118	0.6 (0.3–1.4)
	AA	0.01	0.015	1.6 (0.2–11.8)	0.03	2.7 (0.4–20)	0.008	1.03 (0.1–12)	0.039	3 (0.5–18)
	G	0.90	0.90		0.89		0.89		0.90	
	A	0.10	0.10	0.99 (0.6–1.7)	0.11	1.06 (0.6–2)	0.11	1.1 (0.7–1.9)	0.10	0.9 (0.5–1.7)
CAV1	GG	0.835	0.805	Ref.	0.791	Ref.	0.831	Ref.	0.75	Ref.
rs3807987	GA	0.15	0.188	1.23 (0.7–2.2)	0.149	1 (0.5–2.2)	0.161	0.93 (0.5–1.8)	0.197	1.4 (0.7–2.8)
	AA	0.015	0.008	0.5 (0.05–5.2)	0.06	3.8 (0.8–18.2)	0.008	0.6 (0.06–5.4)	0.053	4.1 (0.9–19.7)
	G	0.91	0.91		0.87		0.91		0.85	
	A	0.09	0.09	1.1 (0.6–1.7)	0.13	1.4 (0.8–2.45)	0.09	0.9 (0.5–1.5)	0.15	1.7 (0.96–2.8)
rs7804372	TT	0.58	0.549	Ref.	0.612	Ref.	0.54	Ref.	0.618	Ref.
	AT	0.345	0.406	1.21 (0.8–1.9)	0.269	0.7 (0.4–1.3)	0.395	1.16 (0.7–1.9)	0.303	0.78 (0.4–1.4)
	AA	0.075	0.045	0.7 (0.3–1.8)	0.119	1.5 (0.6–3.8)	0.065	0.87 (0.4–2.2)	0.079	1.12 (0.4–.3.1)
	T	0.75	0.75		0.75		0.74		0.77	
	A	0.25	0.25	0.99 (0.7–1.5)	0.25	1 (0.7–1.5)	0.26	1.03 (0.7–1.5)	0.23	0.9 (0.6–1.4)
rs3757733	TT	0.635	0.609	Ref.	0.597	Ref.	0.581	Ref.	0.645	Ref.
	AT	0.355	0.376	1.1 (0.7–1.75)	0.403	1.18 (0.7–2.1)	0.403	1.2 (0.8–1.9)	0.355	0.97 (0.6–1.7)
	AA	0.01	0.015	1.4 (0.2–10.4)	0.00	NA	0.016	1.4 (0.2–10.3)	0.000	NA
	T	0.81	0.80		0.80		0.78		0.82	
	A	0.19	0.20	1.12 (0.7–1.7)	0.20	1.1 (0.6–1.8)	0.22	1.2 (0.8–1.9)	0.18	0.9 (0.5–1.6)
rs3807992	GG	0.58	0.579	Ref.	0.612	Ref.	0.565	Ref.	0.632	Ref.
	AG	0.345	0.331	0.9 (0.6–1.5)	0.269	0.7 (0.4–1.3)	0.347	0.98 (0.6–1.6)	0.25	0.63 (0.3–1.2)
	AA	0.075	0.09	1.3 (0.6–2.9)	0.119	1.5 (0.6–3.9)	0.089	1.16 (0.5–2.7)	0.118	1.52 (0.6–3.8)
	G	0.75	0.74		0.75		0.74		0.76	
	A	0.25	0.26	1.1 (0.7–1.5)	0.25	0.97 (0.7–1.4)	0.26	1.04 (0.7–1.5)	0.24	0.97 (0.6–1.5)
rs1997623	CC	0.72	0.654	Ref.	0.761	Ref.	0.702	Ref.	0.671	Ref.
	AC	0.27	0.323	1.3 (0.9–2.1)	0.194	0.7 (0.3–1.4)	0.266	1 (0.6–1.68)	0.303	1.17 (0.7–2.1)
	AA	0.01	0.023	2.5 (0.4–16)	0.045	5.7 (0.9–37)	0.032	4.4 (0.8–25.5)	0.026	2.55 (0.3–19)
	C	0.855	0.82		0.86		0.835		0.82	
	A	0.145	0.18	1.35 (0.9–2.1)	0.14	1.02 (0.6–1.8)	0.165	1.2 (0.8–1.9)	0.18	1.26 (0.8–2.1)
rs12672038	GG	0.805	0.805	Ref.	0.836	Ref.	0.815	Ref.	0.816	Ref.
	GA	0.17	0.188	1.06 (0.6–1.9)	0.104	0.5 (0.2–1.33)	0.177	0.92 (0.5–1.7)	0.132	0.8 (0.4–1.7)
	AA	0.025	0.008	0.3 (0.03–2.6)	0.06	2.06 (0.5–8.2)	0.008	0.3 (0.04–1.3)	0.053	2.1 (0.5–8.6)
	G	0.89	0.90		0.89		0.90		0.88	
	A	0.11	0.10	0.9 (0.5–1.4)	0.11	0.99 (0.6–1.7)	0.10	0.8 (0.5–1.4)	0.12	1.07 (0.6–1.8)

Table 3 (continued)

Variation	Geno-type/ allele	Controls N = 200	Low grade N = 133	OR (95% CI)	High grade N = 67	OR (95% CI)	NMIBC N = 124	OR (95% CI)	MIBC N = 76	OR (95% CI)
NOS3	bb	0.76	0.684	Ref.	0.791	Ref.	0.693	Ref.	0.76	Ref.
	ba	0.205	0.263	1.42 (0.8–2.4)	0.209	1 (0.5–1.99)	0.25	1.3 (0.8–2.3)	0.24	1.2 (0.6–2.3)
	aa	0.035	0.053	1.5 (0.5–4.5)	0	NA	0.057	1.6 (0.5–4.7)	0.00	NA
	b	0.86	0.82		0.90		0.82		0.88	
	a	0.14	0.18	1.3 (0.9–2)	0.10	0.8 (0.4–1.37)	0.18	1.3 (0.9–1.9)	0.12	0.9 (0.5–1.5)
TNFA	GG	0.84	0.902	Ref.	0.776	Ref.	0.871	Ref.	0.842	Ref.
rs1800629	GA	0.15	0.09	0.6 (0.3–1.2)	0.209	1.5 (0.7–3.1)	0.121	0.9 (0.5–1.9)	0.145	0.9 (0.4–1.9)
	AA	0.01	0.008	0.7 (0.06–8)	0.015	1.4 (0.12–16)	0.008	0.9 (0.1–10.2)	0.013	1.01 (0.1–11.5)
	G	0.915	0.95		0.88		0.93		0.914	
	A	0.085	0.05	0.62 (0.3–1.2)	0.12	1.4 (0.8–2.7)	0.07	0.9 (0.5–1.7)	0.086	0.9 (0.5–1.8)
PPARG	CC	0.77	0.782	Ref.	0.702	Ref.	0.758	Ref.	0.75	Ref.
rs1801282	CG	0.215	0.196	0.9 (0.5–1.54)	0.224	1.05 (0.5–2.1)	0.218	1 (0.6–1.8)	0.184	0.8 (0.4–1.6)
	GG	0.015	0.023	1.6 (0.3–8.06)	0.075	**5.97 (1.3–26)**	0.024	1.6 (0.3–8.2)	0.066	**5.4 (1.2–24)**
	C	0.88	0.88		0.81		0.87		0.84	
	G	0.12	0.12	0.98 (0.6–1.6)	0.19	1.5 (0.9–2.6)	0.13	1.1 (0.7–1.7)	0.16	1.3 (0.8–2.4)

Statistically significant values ($p \leq 0.05$) are presented in bold format

RM recessive model, *OR (95% CI)* odds ratio (95% confidence interval) adjusted for age, gender and smoking, *Ref.* reference genotype, *NA* not applicable (rs2567206) of *CYP1B1*, predisposed smokers to greater risk of the disease, which is plausible given the role of this enzyme in carcinogen metabolism and the higher exposure of smokers to cigarette carcinogens. The present study is the first to report an association of this variant with UBC. In two independent studies to determine the role of this SNP in promoter activity, a strong reduction in promoter activity was seen associated with the 'C' allele as compared to 'T' by Chakrabarti et al. [11] in a trabecular meshwork (TM3) cell line, while no effect was observed in a human bronchial epithelial cell line [27]. These contrasting effects are possibly due to tissue-specific expression and transcription factors. As currently there are no reports of the expression association of this variant in bladder epithelium therefore, functional characterization of this SNP in this tissue needs further investigation.

Glutathione-sulfo-transferases (GSTs) are a superfamily of phase-II carcinogen metabolizing enzymes involved in the detoxification of reactive carcinogenic metabolites to less reactive and more hydrophilic compounds. Their mechanism of action involves catalysis of the conjugation of glutathione with electrophilic carcinogen metabolites by forming a thioether bond. The resultant products are less hydrophobic and can be easily excreted [29]. *GSTT1* (encodes GSTθ) and *GSTM1* (encodes GSTμ) are two important members of this superfamily. Each of these harbor a null gene polymorphism (*GSTT0* and *GSTM0*, respectively) causing a loss of enzymatic activity and hence an increased vulnerability to cancer due to inefficient detoxification of carcinogenic metabolites resulting in an increased rate of DNA damage [48]. The role of *GSTT1* null polymorphism in bladder carcinogenesis has been found to be inconsistent [1, 34]. In the present study, a non-significant relation of *GSTT1* polymorphism with UBC predisposition was observed in overall as well as smoking-status based analysis. *GSTM1* polymorphism showed an increased overall risk of UBC and this risk predisposition was irrespective of the smoking-status in agreement with a previously reported meta-analysis [23]. A related possibility is that in addition to tobacco carcinogen metabolism, GSTμ also provides protection against reactive oxygen species, thereby playing a role in smoking- as well as nonsmoking-associated UBC [23]. Upon stratification by tumor grade and stage, *GSTT1* polymorphism was found to be significantly associated with advanced disease, i.e., with high grade and MIBC, while *GSTM1* polymorphism was associated with low grade and NMIBC. Reason of this differential behavior of the two *GSTs* in disease severity can be explained by the following observations. *GSTT1* null polymorphism is particularly associated with a greater background rate of large-scale genetic alterations such as sister chromatid exchange and formation of micronuclei as compared to *GSTM1* [43]. Since the high grade invasive bladder tumors are genetically more unstable and accumulate larger number of genetic alterations than low grade noninvasive UBC [35], therefore *GSTT1* deletion is more likely to be deleterious for the more aggressive group as compared to the less aggressive form of the disease.

GSTμ expressed by *GSTM* is known to inhibit apoptosis via a mechanism independent of its glutathione-conjugating activity; intact GSTμ binds with apoptosis signal-regulating kinase 1 (ASK1) and inhibits its activity [14]. Under stress conditions ASK1 is released from GSTμ and activates kinases that induce apoptosis. Individuals deficient in GSTμ activity have higher ASK1 activity and subsequently greater apoptotic potential than persons with intact GSTμ who have greater chances of proliferation leading to progression [16]. Therefore GSTμ-deficient individuals are less likely to be at an increased risk of developing high grade invasive cancer.

p53 (encoded by *TP53*) is a tumor suppressor protein that is activated following DNA damage, aberrant growth signals or other stresses on the cell and results in regulation of cell cycle, cell proliferation, DNA repair and apoptosis [60]. Part of p53 functions are mediated by its regulation of the expression of p21 (encoded by *CDKN1A*), which in turn binds with cyclin-CDK complexes and induces cell cycle arrest, thus regulating cell proliferation [60]. Two nonsynonymous polymorphisms in these genes including rs1042522 (p.Pro72Arg) of *TP53* and rs1801270 (p.Ser31Arg) of *CDKN1A* have been widely studied in relation to their association with bladder cancer [13, 64; Zhang et al. 64]. Pro72 of p53 and/or 31Arg of p21 have been reported to be associated with lower downstream expression of p21 [57]. In a previous study the 31Arg allele was not found to cause a loss of tumor suppressor activity of p21 [12]. In the present analysis no association was found between these variants and UBC risk in an overall as well as stratified analysis. However, a significant protective effect of 31Arg of p21 ('A' allele) was observed among smokers. Contrary to this Taghavi et al. [58] have reported an increased risk associated with 31Arg for esophageal squamous cell carcinoma among smokers. Cigarette smoke causes DNA damage [36], which may activate p53/p21 pathway since cigarette smoke has been shown to induce p21 expression in vitro and in animal models [59]. Cigarette smoking and overexpression of p53/p21 have been found to be associated with poor prognosis in non-small cell lung cancer [62]. In addition, p21 triggers replicative senescence [33] and in vitro expression of p21 has been shown to trigger enhanced oxidative stress [38]. Also, p21 has a dual role in carcinogenesis by exhibiting both tumor suppressor as well as oncogenic activities [51]. So the biological roles of p21 are not only diverse but are also ambiguous. Further, they are mediated by different factors including its expression levels, transcriptional control, transcript stability and post-translational regulation [25]. According to HOPE pathogenicity prediction, serine is smaller and polar while arginine is comparatively bigger and positively charged. This change in the size and charge of residue might affect protein function. The intriguing finding in the current study prompts investigation into further trying to understand the association between cigarette smoking and this important pathway in relation to cancer susceptibility.

Another protein participating in cell proliferation regulation is insulin-like growth factor binding protein 3 (IGFBP3), which is a member of a superfamily consisting of at least six well-characterized IGFBPs. IGFBP3 participates in cancer prevention by competitively inhibiting the binding of insulin-like growth factors (IGFs) with their targets and subsequent reduction in IGFs' mitogenic effects as well as independently by regulating cell multiplication and by inducing apoptosis [21]. Serum levels of IGFBP3 have been shown to be correlated with susceptibility to bladder cancer in an inverse relationship [53, 66]. An *IGFBP3* SNP (rs2854744) due to its location near the basal promoter activity element is known to modulate *IGFBP3* expression and hence its serum levels; in this SNP the 'A' allele results in higher plasma IGFBP3 levels as compared to the 'C' allele [17]. This imparts a potential protective role to 'A' allele individuals in reducing UBC risk, which was evident in the present study as the 'A' allele-harboring individuals were found to have reduced overall UBC susceptibility as well as reduced risk of high-grade tumor. These results are consistent with those of Safarinejad et al. [53] who correlated the genotype results with IGFBP3 serum levels as well and found AA genotype to confer protection. No previous study was found even upon an extensive literature search of correlation of *IGFBP3* and cigarette smoking with reference to bladder tumor. Here a protective role of AA and CA genotypes of IGFBP3 is reported for the first time among non-smokers and a loss of protective effect among smokers. Smoking has been found to be associated with reduction in serum IGFBP3 levels in men [49] possibly explaining the non-association among smokers in the current study. Association with high grade tumor could be attributed to the potential role of IGFBP3 in the differentiation of bladder cells. Given its relationship with UBC susceptibility and circulating IGFBP3 levels, rs2854744 can be considered an important potential predictor.

Leptin (encoded by *LEP*) is a member of a family of adipose tissue-derived hormones called adipocytokines, and exerts its action through receptors such as leptin receptor (encoded by *LEPR*), which activates specific intracellular pathways. Leptin is primarily involved in energy homeostasis and BMI regulations [31]. Its involvement in carcinogenesis comes from the observation of increased chances of cancer among obese people [10] as well as the role of leptin in cell proliferation, apoptosis, growth regulation and neoangiogenesis [24]. The role of adipocytokines in carcinogenesis has been mainly described through in vitro expression studies and genetic variation analyses are quite scarce [44]. The current study is the first one to determine the possible contribution of two polymorphisms of this pathway in bladder oncogenesis: a promoter SNP (rs7799039) of *LEP* and a nonsynonymous SNP (rs1137101, p.Gln223Arg) of *LEPR*. The former affects leptin secretion and its strength of forming complexes with a nuclear protein [30], while the amino acid at 223 position of leptin receptor is a part of its extracellular domain and a substitution of Gln by Arg is known to enhance serum leptin-binding affinity [46]. In the current study, rs7799039 of *LEP* did not show any association, while a strong association of 223Arg isoform ('G' allele) of *LEPR* with overall UBC susceptibility was observed. As the 223Arg isoform (rs1137101) has a higher affinity for leptin [46], therefore any changes in its structure may act by promoting leptin-mediated growth responses and thus increasing UBC susceptibility. Upon stratified analysis by smoking status, this significant association was only observed among non-smokers which may be explained by the fact that cigarette smoking is associated with lower concentration of leptin [50] thus minimizing the role of rs1137101 among smokers.

We also found rs1137101 to be associated with low grade UBC and NMIBC and no association was observed with high grade or MIBC. Phosphoinositide 3-kinase (PI3K) pathway is frequently activated by mutations in low grade NMIBC and very rarely in high grade MIBC [35]. Leptin is also known to activate PI3K pathway [22], which could be the likely reason behind association of leptin receptor SNP only with low grade and NMIBC.

HOPE analysis predicts an alteration in the charge of residue from neutral (Glu) to positive (Arg) which might affect the binding of ligands. Further the arginine is bigger and might lead to changes in surface geometry of leptin receptor and may affect its interaction with other molecules.

The current novel finding of an association of rs1137101 of *LEPR* provides a potential candidate for future research and may also help to explore the link between obesity and carcinogenesis.

Angiotensin I-converting enzyme (ACE) is a zinc metalloproteinase that apart from its diverse physiological roles, is also known to promote tumor growth, angiogenesis and metastasis [9]. A 187 bp *Alu* repeat sequence in intron 16 of *ACE* has been implicated in the etiology of different cancers [42, 56], which effect has been reported to be achieved by regulating ACE levels. The DD homozygotes have about twofold higher levels of the enzyme as compared to II homozygotes while the I/D heterozygotes have intermediate levels [41]. However, a meta-analysis of different cancers revealed

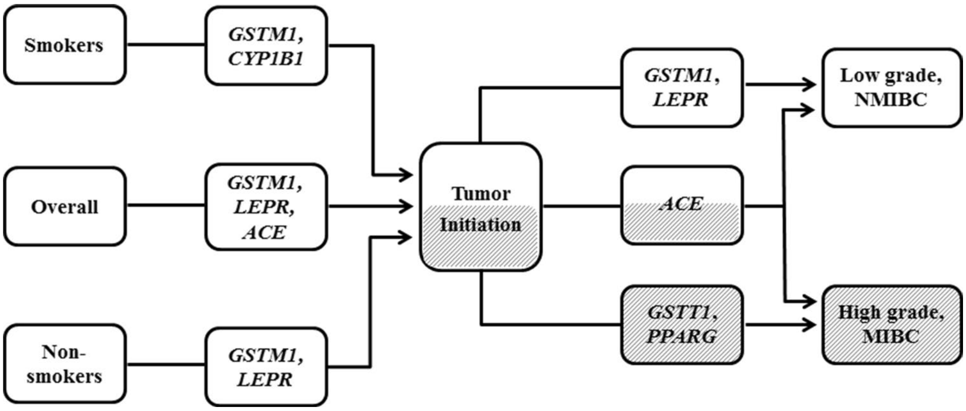

Fig. 1 Association of genes (with respect to selected polymorphisms) with increased risk of overall urothelial bladder carcinoma susceptibility, smoking status-based sub-groups and histopathological subtypes; genes with non-associated polymorphism(s) or those conferring protection are not shown

non-association of this polymorphism with cancer risk [65]. To date, no study has been conducted on bladder cancer and *ACE* I/D polymorphism. In the current study, DD homozygotes were found to confer an increased risk towards overall UBC susceptibility as well as towards low and high grades of cancer and also towards NMIBC and MIBC stages indicating a possible association of ACE activity with underlying mechanisms for different pathological types of UBC.

Peroxisome proliferator activated receptor (PPAR) is a sub-family of nuclear receptors superfamily and includes PPARα, PPARβ/δ and the most widely studied PPARγ [19]. After binding to ligands and coactivators, PPARγ (encoded by *PPARG*) induces the expression of target genes [39]. rs1801282 (p.Pro12Ala) is the most extensively studied polymorphism of *PPARG* and 12Ala isoform ('G' allele) causes a reduction in receptor activity [18]. Pathogenicity prediction using HOPE suggests a change in protein structure as a result of this substitution. Alanine is smaller in size and might lead to a loss of interactions. In addition, proline induces a special backbone conformation which might be required for this position. Substitution by alanine can disturb this conformation and thus protein activity. Inhibition/reduction of PPARγ activity has been found to be associated with higher grade and advanced stages of bladder cancer [63]. In the present study, the 12Ala isoform was found to significantly enhance tumor severity by correlating with high grade and MIBC. PPARγ mediates several processes such as apoptosis, anti-inflammatory effects, terminal differentiation and subverting of host immune response, which are required for prevention of cancer progression, invasion and metastasis [39]. Therefore, a reduction in its activity due to 12Ala is more likely to affect high grade and MIBC. More specifically, the PPARγ agonists have been shown to play an important role in inducing differentiation of bladder cancer cells by causing an increased expression of adipose-type fatty acid binding proteins (A-FABP) [26]. Therefore, the loss of PPARγ activity has been reportedly associated with UBC progression and severity [39, 63].

In brief, association of SNPs with UBC susceptibility and/or severity was observed in selected genetic variants of *CYP1B1, GSTT1* and *GSTM1* genes from carcinogen metabolism and antioxidant pathways, *CDKN1A* from cell cycle regulation pathway, *IGFBP3* and *LEPR* from growth regulation pathway, *ACE* from angiogenesis pathway and transcription regulator *PPARG*.

Rest of the selected polymorphisms did not exhibit any statistically significant association in the current investigation, these were *GSTP1* and *PON1* from carcinogen metabolism and antioxidant pathways, respectively, *VEGFA* from angiogenesis pathway, *MTHFR* from folate metabolism pathway, *CAV1* from cell signaling pathway, *NOS3* from nitric oxide metabolism pathway and inflammatory cytokine *TNFA*.

Association of variants from different pathways demonstrates the heterogeneous genetic etiology evident not only in the overall UBC susceptibility but also in different histopathological subtypes and in relation to smoking. Non-association of certain variants offers a perspective to explore other candidate factors from these pathways.

Moreover, distinct correlations of clinicopathological subtypes of UBC were also observed. Low grade UBC is frequently known to occur with NMIBC while high grade with MIBC, representing two distinct disease entities with distinct underlying molecular mechanisms and behaviors [35]. Concordant with this, we also found the co-occurrence of low grade with NMIBC (48% of the cases) and of high grade with MIBC (28% of the cases), the rest 24% were either low grade MIBC or high grade NMIBC. Further, the polymorphisms either associated with low grade, NMIBC or with high grade, MIBC with the exception of ACE, which associated with both groups although conferring slightly greater risk for high grade and MIBC (Fig. 1). Thus further studies are required to identify specific sets of prognostic and susceptibility markers for these two groups.

Strengths of the present study are a selection of polymorphisms from multiple pathways based on previous reports as well as predicted involvement and a stratified analysis by smoking status, tumor grade and tumor stage. In addition, this is a preliminary report from Pakistani UBC cases. A major limitation was the sample size due to the relatively low prevalence and/or reporting rate in Pakistan, poor cooperation of indoor patients and a lack of follow-up cases.

Conclusion

The present study is the first attempt to determine an association of selected common variants with UBC in the Pakistani population. In the current study there are a few novel findings and others are a validation of previous ones in different populations. Identification of some putative novel association indicates potential involvement of different pathways in the disease pathology. It also warrants further studies to better understand the molecular mechanisms underlying this multifactorial disorder. Since this was a preliminary study of the Pakistani population, future studies with larger sample size could be helpful to validate these findings and to determine their functional effects. Expression-based studies would be helpful to assess the prognostic significance of some of the variants associated with aggressive tumor and towards personalized therapeutic interventions.

Acknowledgements This study was sponsored by Pakistan Academy of Sciences (Grant No. 5-9/PAS awarded to RQ) and Radboud University Medical Center, Nijmegen, The Netherlands.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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