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ERCC1 haplotypes modify bladder cancer risk: A case–control study

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ABSTRACT

Bladder cancer risk is highly influenced by environmental and/or predisposing genetic factors. In the last decades growing evidence of the major role played by DNA repair systems in the developing of bladder cancer has been provided. To better investigate the involvement of DNA repair genes previously reported to be significantly associated with bladder cancer risk, we examined in a case–control study (456 cases and 376 hospital controls) 36 single nucleotide polymorphisms (SNPs) in 10 DNA repair genes, through a better gene coverage and a deep investigation of the haplotype role. A single SNP analysis showed a significantly increased risk given by *XRCC1*-rs915927 G allele (OR = 1.55, CI 95% 1.02–2.37 for dominant model) and a protective effect of the rare alleles of 3 *ERCC1* SNPs: rs967591 (OR = 0.66, CI 95% 0.46–0.95), rs735482 (OR = 0.62, CI 95% 0.42–0.90) and rs2336219 (OR = 0.63, CI 95% 0.43–0.93). Haplotype analysis revealed that cases had a statistically significant excess of *XRCC3*-TAGT and *ERCC1*-GAT haplotypes, whereas *ERCC1*-AAC, *MGMT*-TA, *XRCC1*-TGCC and *ERCC2*-TGAA haplotypes were significantly underrepresented. Together with other published data on large case–control studies, our findings provide epidemiological evidence supporting a link between DNA repair gene variants and bladder cancer development, and suggest that the effects of high-order interactions should be taken into account as modulating factors affecting bladder cancer risk. A detailed characterization of DNA repair genetic variation is warranted and might ultimately help to identify multiple susceptibility variants that could be responsible for joint effects on the risk.

1. Introduction

Bladder cancer is the most common cancer of the urinary tract and it is the eighth most common cancer among men, with about 330,000 new incident cases per year in the world [1]. The incidence of bladder cancer varies considerably among countries [2]; in general, the highest incidence rates for bladder cancer are in South-Western Europe, North America and Australia. In Turin the incidence rates is quite high (38.5 × 100.000 incidence rate, world standardization, year 1998–2002), comparable with other Italian and Southern European cities. Bladder cancer incidence increases with age, occurring rarely before the age of 40. Due to the ageing of developing countries populations, the burden of bladder cancer will further increase in the next decades.

Bladder cancer is associated with exposure to tobacco [3] and occupational exposure [4] and there are suggestions about the involvement of predisposing genetic factors.

Environmental and occupational chemical carcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines and *N*-nitroso compounds, form DNA adducts repaired primarily through the nucleotide excision repair (NER) pathway (e.g. Excision Repair Cross-Complementing rodent repair deficiency, complementation group 1–2 (*ERCC1–2*) genes and Xeroderma Pigmentosum, complementation group A–C (*XPA–XPC*) genes). Those agents can produce interstrand cross-links repaired by genes involved in NER (e.g. *ERCC1-2-4*), in homologous recombinational repair (HR) (e.g. X-ray Repair Cross-Complementing group 1 (*XRCC1*) and APEX nuclease (*APEX1*)) or in double-strand break repair (DSBR) (e.g. *XRCC3*) pathways.

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Table 1
List of genes/polymorphisms analysed with number of subjects genotyped for each polymorphism.

Chr	Location	Gene	Function	SNP common name	rs	Variants	N typed Ctrs/Cases	% typed Ctrs/Cases	Technique
3	3p25	*XPC*	NER	XPC rs2228001	2228001	A>C	286/328	76.06/71.93	TQ
				XPC PAT	Ins/Del	Ins/Del	174/151	46.28/33.11	PCR
9	9q22.3	*XPA*	NER	XPA 23 A/G	1800975	G>A	313/320	83.24/70.18	TQ
10	10q26	*MGMT*	Direct reversal	MGMT 127215 C/T	12917	C>T	298/349	79.26/76.54	TQ
				MGMT rs 2308321	2308321	A>G	197/180	52.39/39.47	TQ
				MGMT 185571 A/G	2308327	A>G	14/23	3.72/5.04	TQ
14	14q11.2	*APEX1*	BER	APEX 1738 T/G	1130409	T>G	296/346	78.72/75.88	TQ
	14q32.3	*XRCC3*	DSBR/HR	XRCC3 18067 C/T (ex 7)	861539	C>T	374/453	99.47/99.34	DG/DH/TQ
				XRCC3 17893 A/G	1799796	A>G	370/441	98.40/96.71	TQ
				XRCC3 rs 861531	861531	G>T	284/327	75.53/71.71	TQ
				XRCC3 rs 861530	861530	G>A	289/327	76.86/71.71	TQ
				XRCC3 4541 C/T	1799794	T>C	230/275	61.17/60.31	DH
16	16p13.3	*ERCC4*	NER	ERCC4 30028 C/T Ser835Ser	1799801	T>C	161/197	42.82/43.20	PEX
				ERCC4 2020957 A/G Ile873Val	2020957	A>A	161/196	42.82/42.98	PEX
				ERCC4 30147 G/A Glu875Gly	1800124	A>G	220/260	58.51/57.02	PEX/DH
19	19q13.2	*XRCC1*	BER	XRCC1 28152 G/A (ex 10)	25487	G>A	368/446	97.87/97.81	TQ/DG
				XRCC1 26651 G/A	915927	A>G	289/333	76.86/73.02	TQ/DH
				XRCC1 26304 T/C	1799782	C>T	362/448	96.28/98.25	TQ/DH
				XRCC1 rs762507	762507	G>A	289/329	76.86/72.15	TQ
				XRCC1 rs1799778	1799778	C>A	285/328	75.80/71.93	TQ
				XRCC1 rs2854501	2854501	C>T	274/312	72.87/68.42	TQ
				XRCC1 rs1001581	1001581	C>T	284/322	75.53/70.61	TQ
				XRCC1 rs2854509	2854509	C>A	290/328	77.13/71.93	TQ
				XRCC1 rs3213255	3213255	T>C	289/326	76.86/71.49	TQ
	19q13.3	*ERCC2*	NER	XPD 35931 C/A (ex 23)	13181	A>C	371/450	98.67/98.68	TQ/DG
				ERCC2 rs1052555	1052555	C>T	240/292	63.83/64.04	TQ
				ERCC2 rs1799787	1799787	C>T	277/313	73.67/68.64	TQ
				ERCC2 rs3916874	3916874	G>C	258/301	68.62/66.01	TQ
				ERCC2 rs171140	171140	A>C	286/320	76.06/70.18	TQ
				XPD 23591 G/A (ex 23)	1799793	G>A	361/432	96.01/94.74	TQ
	19q13.3	*ERCC1*	NER	ERCC1 rs967591	967591	G>A	288/325	76.60/71.27	TQ
				ERCC1 rs735482	735482	A>C	283/324	75.27/71.05	TQ
				ERCC1 rs2336219	2336219	G>A	275/323	73.14/70.83	TQ
				ERCC1 rs3212955	3212955	A>G	278/322	73.94/70.61	TQ
				ERCC1 19007 C/T	11615	T>C	323/385	85.90/84.43	TQ
20	20p12	*PCNA*	DNA PS	PCNA 6084 G/C	3626	G>C	357/430	94.95/94.30	TQ/DH

Reactive oxygen species can also induce base damage, abasic sites, single-strand breaks and double-strand breaks: single-strand breaks are repaired through the base excision repair (BER) pathway (e.g. *XRCC1* and proliferating cell nuclear antigen (*PCNA*)), whereas double-strand breaks are corrected by either homologous recombination (e.g. *XRCC3*) or non-homologous end-joining pathways.

Hundreds of polymorphisms in DNA repair genes have been identified; however, the effect on repair phenotype and cancer susceptibility remains uncertain for many of these (see also dbSNP database: http://www.ncbi.nlm.nih.gov/SNP/ and dbGaP: http://www.ncbi.nlm.nih.gov/gap).

In the present study, we have more thoroughly investigated the most interesting and significant results we previously published [5,6] expanding our DNA repair gene polymorphisms analyses to 36 biallelic polymorphisms in ten DNA repair genes involved in different repair pathways. Furthermore, we examined the bladder cancer risk associated with estimated phased haplotypes for SNPs lying in the same gene, including many tag-SNPs and possible functional SNPs for the most significant genes. An analysis of epistasis has also been carried out.

2. Materials and methods

2.1. Subjects

We conducted a hospital-based case–control investigation at S. Giovanni Battista hospital in Turin, where about half of the incident bladder cancer in the Turin metropolitan area are treated.

The case group comprises men, aged 40–75 years, resident in the Turin metropolitan area with a newly diagnosed histologically confirmed bladder cancer, treated in the three urology departments of that hospital from 1994 to 2008. Cases were identified by daily contact between a trained interviewer and the urology departments and included in the study after histological confirmation from the pathology department.

The control group comprises men of the same age and residence that are recruited daily in random fashion from patients treated at the same urology departments for non-neoplastic disease (prostatic hyperplasia, cystitis and others) or from patients treated at the medical and surgical departments for hernias, vasculopathies, diabetes, heart failure, asthma or other benign diseases. Patients with cancer, liver or renal diseases and smoking-related conditions were excluded and all diseases were represented in 10% of controls maximum.

All subjects in the study signed an informed consent form.

Before treatment, a trained interviewer used a detailed questionnaire to conduct a face-to-face interview with all subjects on their history of tobacco smoking (including brands and tobacco type), occupational history and a 24-h medication use recall. Additionally, for subjects recruited after 1999, we use a 22-item food frequency questionnaire to assess food consumption.

Blood samples were collected before therapy and stored in ethylenediaminetetraacetic acid (EDTA).

2.2. DNA repair gene polymorphisms

White blood cell (WBC) DNA was isolated and purified from stored buffy-coat samples by enzymatic digestion of RNA and proteins, followed by phenol–chloroform extraction for the first third of the collected sample [7] and by salting-out for the rest of sample [8].

We used a variety of genotyping techniques, choosing the most efficient approaches (i.e. reliable and cost-effective) for any given SNP.

A list of the studied polymorphisms and the related technique used is reported in Table 1. Most of the polymorphisms (rs2228001, rs1800975, rs12917, rs2308321, rs2308327, rs1130409, rs1799796, rs861531, rs861530, rs762507, rs1799778, rs2854501, rs1001581, rs2854509, rs3213255, rs1052555, rs1799787, rs3916874, rs171140, rs1799793, rs967591, rs735482, rs2336219, rs3212955, rs11615) were genotyped with the 5′ Nuclease assay (TaqMan) with fluorogenic Minor Groove Binder probes. The remaining polymorphisms were genotyped partly with TaqMan and partly with PCR–RFLP (rs861539, rs25487, rs13181), Primer Extension/Denaturing High-Performance Liquid Chromatography (rs861539, rs1800124, rs915927, rs1799782, rs3626), Primer extension/sequencing (rs1799801, rs2020957, rs1800124), as reported in Matullo et al. [6].

2.3. DNA typing quality control

Methodological validation included a comparison between the PCR-Restriction Fragment Length Polymorphism, DHPLC and TaqMan assays. Moreover, at least 10% of the genotyping was randomly repeated for each polymorphism. Concordance was in the range between 99 and 100% for all the comparisons; discordant genotypes were excluded from the analysis. We also fully repeated the genotyping of two polymorphisms, rs861531 and rs26651, with a concordance of 98% in the first and 100% in the second.

2.4. Statistical analysis

For each SNP we calculated the Odds Ratios (OR) and the corresponding 95% Confidence Interval (95% CI) following four different models: the dominant model (homozigous wild-type genotype – AA – vs heterozigotes – AB – plus homozigous variant genotype – BB), the codominant model (AA vs AB and AA vs BB), the recessive model (AA plus AB vs BB) and the log-additive model (analysis of trend where AA is '0', AB is '1/2' and BB is '1'). We performed crude analyses and we adjusted them for age (considered as a class variable of the quartiles in controls) , smoking status (current smoker, never smokers and former smoker who ceased smoking since at least 1 year) and intake of fruits and vegetables (a variable based on quartiles of cumulative intake in controls). Subjects with missing values for adjustment variables were removed. All these analysis were performed using SNPStats [9].

To identify haplotypes, we performed a Linkage Disequilibrium (LD) analysis in our controls, grouping SNPs by chromosome. We identified putative recombination hotspots and defined different regions. In these regions we used the R^2 to identify markers in strong LD and we chose only one tag SNP when the $R^2 > 0.80$. In each region, we imputed the phase of the haplotype using a Bayesian method in which the prior was based on an approximation to the coalescent and the inference carried out from Markov Chain Monte Carlo (MCMC). For each haplotype we performed a test for difference in order to notice the different representation between cases and controls and we computed the ORs referred to the most represented haplotype in that region.

The analysis of LD and tag SNPs were performed with Haploview 4.1 [10]; the phase of haplotypes was inferred using PHASE 2.1 [11,12].

Table 2
Cases and controls distribution by age, smoking status and intake of fruits and vegetables. *p*-Value were obtained with Student's *t*-test for continuous variables and χ^2-test for categorical variables.

	Cases (%)	Controls (%)	*p*-Value
Age			
Mean	63.78	60.55	<0.0001
S.D.	7.64	7.89	
1st quartile	69 (15.13)	94 (25.00)	
2nd quartile	93 (20.39)	95 (25.27)	
3rd quartile	115 (25.22)	93 (24.73)	
4th quartile	179 (39.25)	94 (25.00)	
Smoke			
Never	25 (5.48)	89 (23.67)	
Former	216 (47.37)	193 (51.33)	
Current	215 (47.15)	94 (25.00)	<0.0001
Fruits and vegetables			
1st quartile	111 (33.53)	73 (25.09)	
2nd quartile	92 (27.79)	73 (25.09)	
3rd quartile	72 (21.75)	73 (25.09)	
4th quartile	56 (16.92)	72 (24.73)	0.02

Age: The 25th-percentile is 55 y/o; the median is 60.94 y/o; the 75th-percentile is 67.18 y/o; Fruits and vegetables: The 25th-percentile is 2.3 portions/week; the median is 3.4 portions/week; the 75th-percentile is 5.1 portions/week.

To account for multiple comparisons, we estimated the False Discovery Rate (FDR) based on the Benjamini–Hochberg method [13] and we computed the FDR-adjusted *p*-Values at 5% level. We also estimated the False Positive Report Probability (FPRP) [14].

To analyse the gene–gene interaction we used the Multifactor Dimensionality Reduction (MDR) method [15]. In order to avoid mistakes due to missing genotypes, we excluded 209 subjects (the first 209 we have collected in our study) that have less than 50% of genotyped SNPs. We also excluded seven SNPs that have less than 70% of genotyped subjects. In the remaining sample, we had less then 3.8% of missing values, so we could analyse our data incorporating this information into the model.

3. Results

We identified 456 male bladder cancer patients and 376 male hospital controls, with these subjects, with a fixed type I error of 0.05, a probability of exposure in controls of 0.3 and an expected OR of 1.5, we have a power of 82%. As it reported in Table 2, the mean in age in cases is 63.38 ± 7.64 years and in controls is 60.55 ± 7.89 years; as expected, smokers are more represented in cases than in controls, while heavy fruits and vegetables consumers are more represented in controls than in cases.

We analysed 36 SNPs in 10 DNA repair genes. All polymorphisms were in Hardy–Weinberg equilibrium both in case and in control groups.

3.1. Single SNP analysis

The ORs for all the models we tested were presented in Table 3. They are adjusted by smoking status, by quartiles of age in controls and by quartiles of intake of fruits and vegetables.

The G-allele of *XRCC1*-rs915927 polymorphism resulted to be positively associated with bladder cancer with an OR = 1.55 (CI 95% 1.02–2.37) for the dominant model and OR = 1.22 (CI 95% 0.93–1.62) for the log-additive model. The rare alleles in three SNPs in gene *ERCC1* (rs967591, rs735482, rs2336219) resulted to be protective for bladder cancer with an OR for the dominant model of 0.66 (CI 95% 0.46–0.95), 0.62 (CI 95% 0.42–0.90) and 0.63 (CI 95% 0.43–0.93), respectively and an OR of 0.70 (CI 95% 0.51–0.96), 0.68 (CI 95% 0.48–0.95) and 0.67 (CI 95% 0.48–0.94), according to the log-additive model. After the adjustment for multiple comparisons the

Table 3
Single SNP analysis; OR adjusted for age, smoking status and intake of fruits and vegetables and 95% confidence interval for different models.

Chr	Gene	rs	N controls/N cases	Codominant (AA vs AB)	Codominant (AA vs BB)	Dominant (AA vs AB plus BB)	Recessive (AA plus AB vs BB)	Log additive	TAG
3	*XPC*	2228001	286/327	1.00 (0.67–1.48)	0.91 (0.55–1.49)	0.97 (0.67–1.42)	0.91 (0.59–1.40)	0.96 (0.75–1.23)	
		PAT	174/151	1.33 (0.74–2.39)	1.33 (0.66–2.66)	1.33 (0.76–2.31)	1.10 (0.62–1.97)	1.16 (0.82–1.64)	
9	*XPA*	1800975	279/316	1.06 (0.73–1.53)	1.17 (0.66–2.08)	1.08 (0.76–1.54)	1.14 (0.67–1.95)	1.07 (0.83–1.40)	
10	*MGMT*	12917	287/324	0.83 (0.55–1.25)	0.44 (0.13–1.54)	0.79 (0.53–1.17)	0.47 (0.13–1.61)	0.78 (0.55–1.11)	TAG
		2308321	197/180	0.75 (0.44–1.27)	1.26 (0.14–11.52)	0.76 (0.45–1.29)	1.35 (0.15–12.29)	0.80 (0.49–1.31)	TAG
		2308327	7/7	0.14 (0.00–12.69) [a]	–	–	–	–	
14	*APEX1*	1130409	286/324	0.93 (0.63–1.37)	0.96 (0.59–1.56)	0.94 (0.65–1.35)	1.00 (0.65–1.54)	0.97 (0.77–1.24)	
	XRCC3	861539	290/328	1.08 (0.74–1.59)	1.26 (0.76–2.10)	1.13 (0.78–1.62)	1.20 (0.77–1.90)	1.12 (0.87–1.43)	TAG
		1799796	289/322	0.87 (0.61–1.25)	0.64 (0.32–1.30)	0.83 (0.59–1.17)	0.68 (0.34–1.36)	0.83 (0.63–1.10)	TAG
		861531	284/327	1.07 (0.73–1.58)	1.35 (0.80–2.27)	1.14 (0.79–1.64)	1.29 (0.81–2.06)	1.15 (0.89–1.47)	TAG
		861530	289/327	0.99 (0.70–1.42)	1.02 (0.54–1.92)	1.00 (0.71–1.41)	1.02 (0.55–1.87)	1.00 (0.77–1.31)	
		1799794	147/151	0.77 (0.45–1.32)	2.20 (0.40–12.25)	0.83 (0.49–1.40)	2.42 (0.44–13.33)	0.93 (0.59–1.48)	TAG
16	*ERCC4*	1799801	153/155	0.92 (0.55–1.55)	0.91 (0.41–2.02)	0.92 (0.56–1.50)	0.95 (0.44–2.02)	0.94 (0.66–1.35)	TAG
		2020957	153/154	Monomorphic SNP					TAG
		1800124	153/155	0.96 (0.32–2.92)[a]	–	–	–	–	TAG
19	*XRCC1*	25487	287/326	1.05 (0.73–1.51)	0.97 (0.56–1.69)	1.03 (0.73–1.46)	0.95 (0.56–1.60)	1.00 (0.78–1.29)	
		915927	222/234	**1.64 (1.04–2.58)**	1.37 (0.77–2.41)	**1.55 (1.02–2.37)**	1.03 (0.62–1.71)	1.22 (0.93–1.62)	
		1799782	281/325	0.69 (0.43–1.10)	NA (0.00–NA)	0.71 (0.45–1.12)	NA (0.00–NA)	0.74 (0.47–1.16)	TAG
		762507	289/329	1.39 (0.96–2.03)	1.01 (0.61–1.66)	1.28 (0.90–1.82)	0.84 (0.53–1.31)	1.07 (0.84–1.36)	TAG
		1799778	285/328	1.07 (0.75–1.55)	0.96 (0.56–1.67)	1.05 (0.74–1.48)	0.93 (0.56–1.56)	1.01 (0.78–1.30)	
		2854501	274/311	1.44 (0.99–2.09)	0.86 (0.34–2.13)	1.37 (0.95–1.96)	0.75 (0.30–1.84)	1.22 (0.89–1.66)	TAG
		1001581	284/322	0.99 (0.68–1.44)	1.05 (0.63–1.75)	1.01 (0.71–1.43)	1.05 (0.66–1.69)	1.02 (0.80–1.30)	TAG
		2854509	290/328	1.24 (0.85–1.81)	0.87 (0.34–2.23)	1.20 (0.83–1.72)	0.81 (0.32–2.07)	1.12 (0.82–1.52)	
		3213255	289/325	1.23 (0.85–1.80)	1.00 (0.61–1.67)	1.17 (0.82–1.67)	0.89 (0.56–1.41)	1.04 (0.82–1.33)	
	ERCC2	13181	289/326	1.03 (0.70–1.52)	0.89 (0.54–1.47)	0.99 (0.69–1.43)	0.88 (0.56–1.36)	0.96 (0.75–1.22)	
		1052555	240/292	1.05 (0.70–1.57)	0.64 (0.36–1.15)	0.94 (0.64–1.38)	0.62 (0.36–1.07)	0.86 (0.65–1.13)	TAG
		1799787	277/313	0.91 (0.62–1.32)	0.67 (0.40–1.15)	0.84 (0.59–1.20)	0.71 (0.43–1.16)	0.84 (0.65–1.08)	
		3916874	258/301	1.23 (0.83–1.83)	0.93 (0.41–2.09)	1.18 (0.81–1.72)	0.87 (0.39–1.93)	1.09 (0.81–1.48)	TAG
		171140	286/320	1.01 (0.69–1.49)	1.23 (0.74–2.06)	1.06 (0.74–1.53)	1.22 (0.77–1.93)	1.09 (0.85–1.40)	TAG
		1799793	283/326	0.85 (0.58–1.26)	0.74 (0.45–1.23)	0.82 (0.57–1.18)	0.82 (0.53–1.27)	0.86 (0.67–1.10)	TAG
	ERCC1	967591	288/325	**0.67 (0.46–0.98)**	0.58 (0.21–1.61)	**0.66 (0.46–0.95)**	0.65 (0.23–1.81)	**0.70 (0.51–0.96)**	
		735482	283/324	**0.60 (0.40–0.89)**	0.82 (0.25–2.67)	**0.62 (0.42–0.90)**	0.94 (0.29–3.03)	**0.68 (0.48–0.95)**	
		2336219	275/323	**0.64 (0.43–0.94)**	0.59 (0.18–1.98)	**0.63 (0.43–0.93)**	0.66 (0.20–2.23)	**0.67 (0.48–0.94)**	TAG
		3212955	278/322	1.02 (0.71–1.46)	1.62 (0.77–3.42)	1.08 (0.77–1.54)	1.61 (0.78–3.35)	1.14 (0.86–1.50)	TAG
		11615	289/328	0.77 (0.53–1.13)	0.85 (0.52–1.39)	0.79 (0.55–1.13)	0.98 (0.63–1.53)	0.89 (0.70–1.14)	TAG
20	*PCNA*	3626	286/325	1.36 (0.87–2.13)	3.72 (0.61–22.59)	1.44 (0.93–2.22)	3.49 (0.58–21.13)	1.46 (0.97–2.18)	

[a] Unic model; only the AA and AG genotypes are present.

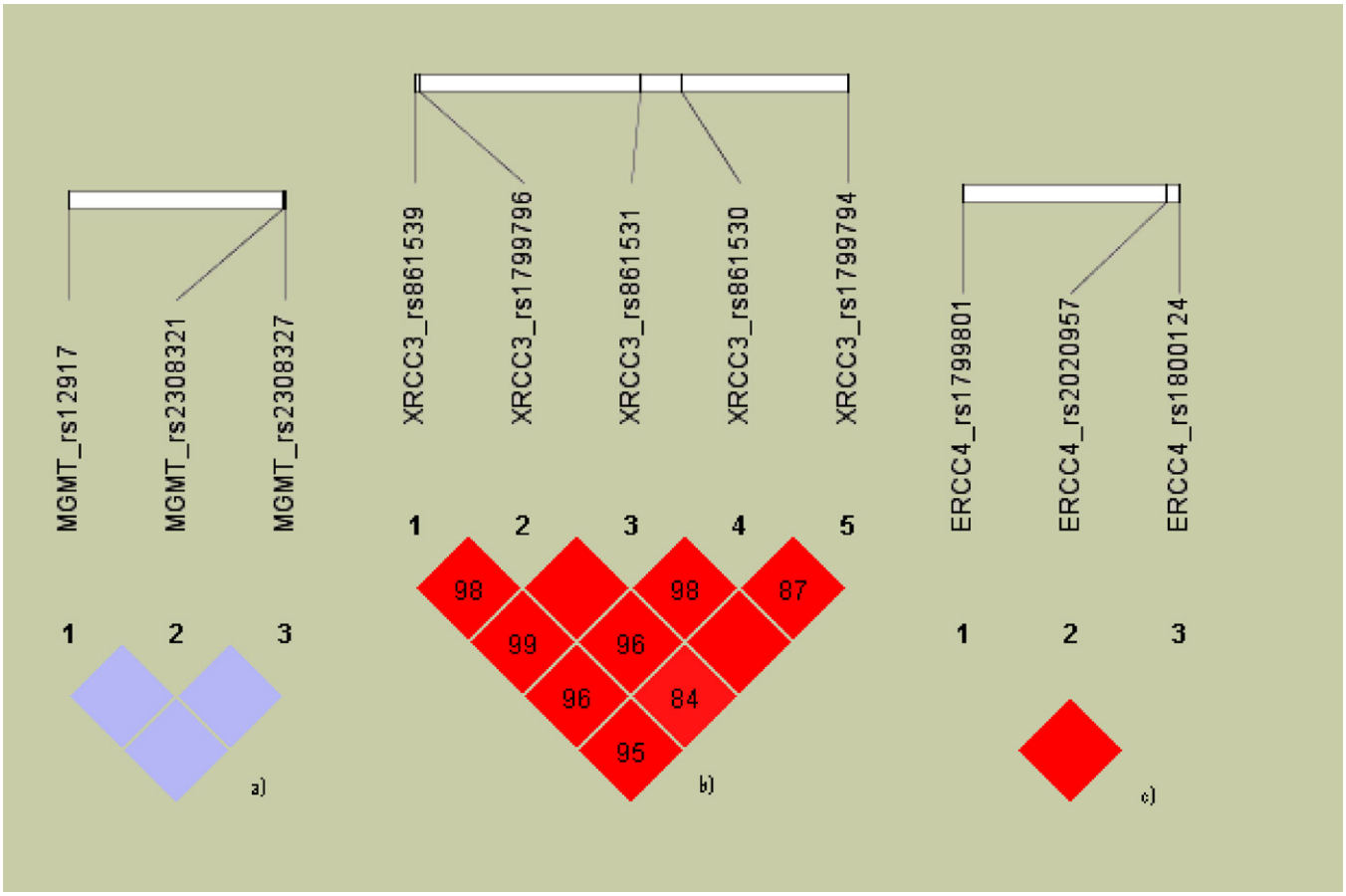

Fig. 1. D′ linkage disequilibrium values (100×) between polymorphisms in the region of gene *MGMT* in chromosome 10q26 (a), gene *XRCC3* in chromosome 14q32.3 (b) and gene *ERCC4* in chromosome 16p13.3 (c).

three ORs for *ERCC1* gene still remained significant (FDR-corrected *p*-Values of 0.028, 0.024 and 0.021 respectively), while the association identified with the SNP in *XRCC1* gene became not statistically significant (FDR-corrected *p*-Value of 0.18).

In the supplemental data (Table S1) we presented the FPRP results.

3.2. Haplotype analysis

Figs. 1 and 2 show the results of the D′ LD analysis in controls. We identified only one block in chromosomes 10, 14 (excluding the polymorphism in *APEX1* gene) and 16 while three different blocks can be reconstructed for the *XRCC1, ERCC2* and *ERCC1* genes. The selected tag SNPs are marked in Table 3: two tag SNPs for the gene *MGMT* on chromosome 10, four for *XRCC3* gene on chromosome 14; all three SNPs for *ERCC4* gene on chromosome 16; on chromosome 19 four SNPs for *XRCC1* gene, four for *ERCC2* and three for *ERCC1* genes.

In Table 4 we present the results of the haplotype analysis. We found some relevant difference in haplotype distribution in cases and in controls. The TAGT haplotype in *XRCC3* gene is significantly more present in cases than in controls and because of the fact that is the only haplotype comprising the rs861539-T allele, that is an important addition to the single SNP analysis. We also found a strong significant difference in TA haplotype in *MGMT* gene, a strong significant difference in TGCC haplotype in *XRCC1* gene and a significant difference in TGAA haplotype in *ERCC2* gene, all more present in controls. The haplotype analysis in *ERCC1* gene still revealed differences: GAT haplotype (the only haplotype with the T allele in rs11615) is significantly more present in cases, while AAC (the only haplotype with the A allele in rs2336219) is significantly more present in controls.

3.3. Gene–gene interaction

From the MDR analysis, the best model for epistasis can be observed in Fig. 3. It suggests an interaction between the rs2336219 SNP of *ERCC1* gene and the rs1799782 SNP of *XRCC1* gene. The Test Accuracy of this model is 0.55 that is border-line significant. The dendrogram (Fig. 3) suggests a synergic relationship between the two SNPs we mentioned before, while the joint effect of these two SNPs is correlated with the rs1799796 SNP of *XRCC3* gene.

4. Discussion

Our study has investigated the relationships between bladder cancer and 36 polymorphisms and their haplotypes in 10 DNA repair genes, belonging to different repair pathways: NER (*XPC, XPA, ERCC1-2-4*), BER (*APEX1, XRCC1, PCNA*), DSBR (*XRCC3*), and direct reversal of damage (DRR) [O-6-methylguanine-DNA methyltransferase (*MGMT*)]. Furthermore, we explored the bladder cancer risk associated with estimated phased haplotypes for SNPs lying in the same gene, including several tag-SNPs and possible functional SNPs for the most significant genes previously reported in our bladder cancer study [5,6].

In molecular epidemiology at least two different ways of identifying associations can be used: genome-wide association studies are conducted with the goal of identifying associations without

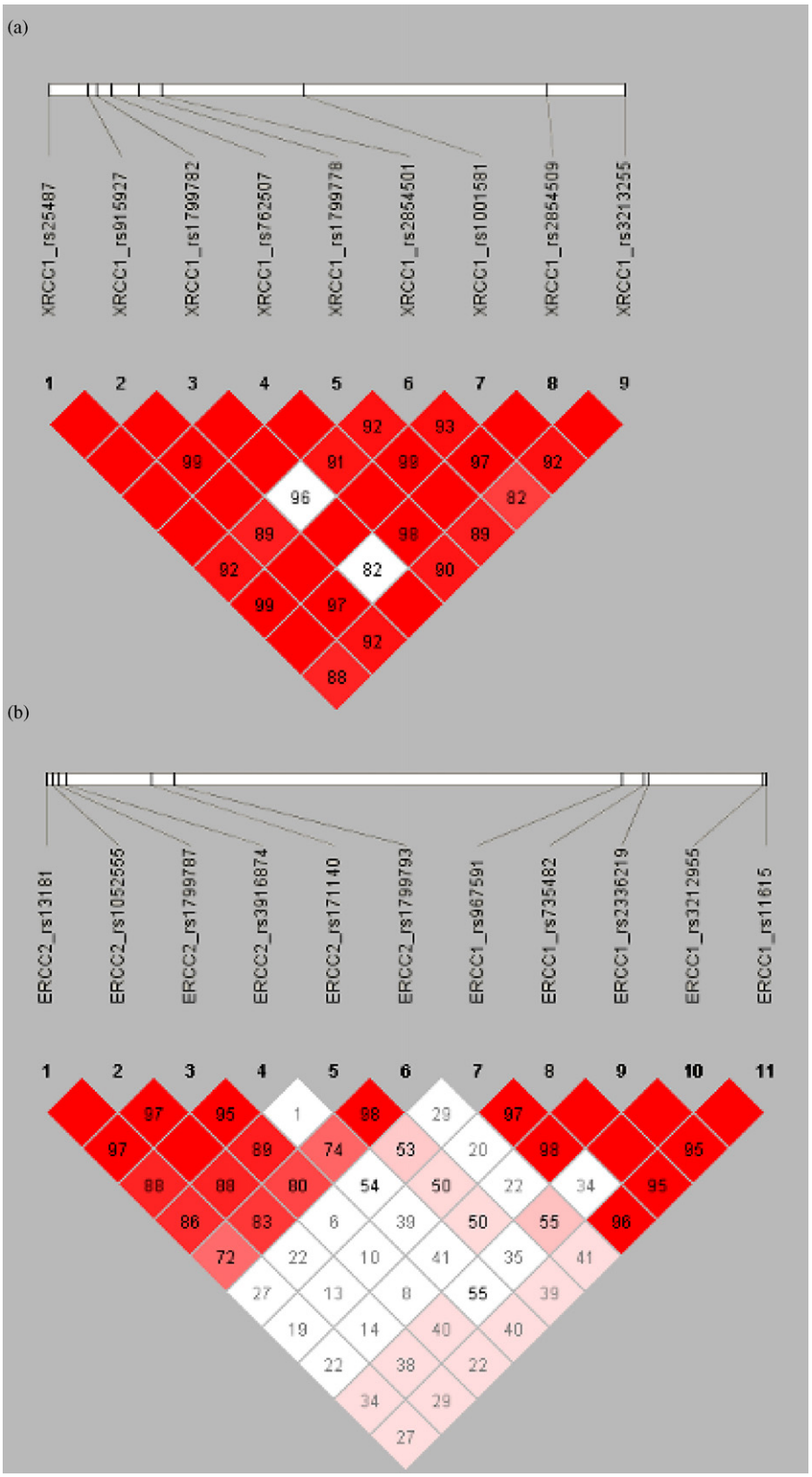

Fig. 2. D′ linkage disequilibrium values (100×) between polymorphisms in the region of gene *XRCC1* in chromosome 19q13.2 (a) and genes *ERCC2* and *ERCC1* in chromosome 19q13.3.

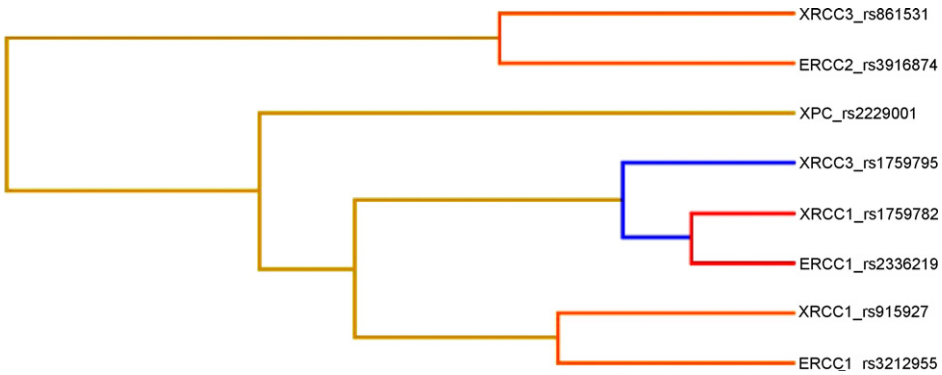

Fig. 3. Dendrogram resulting from MDR analysis. Short line connecting two attributes means strong interaction. Red and orange lines suggest there is a synergistic relationship (i.e. epistasis); yellow lines suggest independence. Green and blue lines suggest redundancy or correlation.

any *a priori* hypothesis while candidate-gene studies are conducted using a priori hypotheses to identify candidate SNPs on which to test a possible association.

The first genome-wide analysis on patients with bladder cancer showed a strongly significant increase of risk in subjects with a sequence variant in 8q24 [16], the same chromosome region that is associated to different other cancers, such as prostate cancer [17], colorectal cancer [18] and breast cancer [19]. The genetic interpretation of these findings is still pending.

DNA repair pathways are quite well characterized and DNA repair gene polymorphisms have been extensively studied in the last years starting from the biological hypothesis that cancer can arise in subjects with low DNA repair capacity [20].

Many studies have been published so far on the association between bladder cancer risk and DNA repair gene polymorphisms (http://www.episat.org/episat/huge/; [21]), but very few emerged to be still significant after meta- and pooled-analyses. A very recent comprehensive meta-analysis considering all published DNA repair polymorphisms and all cancer sites [21] showed that among the studied polymorphisms, *ERCC1* codon 118 (rs11615) and *ERCC2* codon 312 (rs1799793) were nominally significant under the dominant model, although they lost their statistical significance when

Table 4
Haplotype analysis; frequence in cases and controls and difference. OR adjusted for age, smoking status and intake of fruits and vegetables and 95% confidence interval.

	Haplotype	Freq controls (N haplotypes)	Freq cases (N haplotypes)	Difference	OR (95% CI)
Chr 10 *MGMT*	CA	0.825 (480)	0.853 (565)	−0.028	1
	TA	0.142 (82)	0.114 (75)	**0.028****	0.78 (0.70–0.87)
	CG	0.029 (17)	0.030 (20)	−0.001	0.99 (0.65–1.51)
	TG	0.004 (3)	0.003 (2)	0.001	0.76 (0.03–17.88)
Chr 14 *XRCC3*	**TAGT**	0.394 (229)	0.427 (282)	**−0.033***	1
	CGGT	0.252 (147)	0.243 (161)	0.009	0.89 (0.84–0.94)
	CAAC	0.191 (111)	0.187 (124)	0.004	0.90 (0.85–0.96)
	CAAT	0.132 (77)	0.121 (80)	0.011	0.85 (0.77–0.93)
	Rare haplotypes (<1%)	0.031 (18)	0.022 (15)	0.009	0.66 (0.45–0.93)
Chr 16 *ERCC4*	TAA	0.687 (210)	0.675 (209)	0.012	1
	CAA	0.277 (85)	0.300 (93)	−0.023	1.10 (1.03–1.18)
	CAG	0.026 (8)	0.018 (6)	0.008	0.70 (0.32–1.56)
	Rare haplotypes (<1%)	0.011 (3)	0.007 (2)	0.003	0.69 (0.10–4.56)
Chr 19 *XRCC1*	CGCT	0.373 (217)	0.364 (241)	0.009	1
	CATC	0.198 (115)	0.215 (142)	−0.016	1.11 (1.05–1.18)
	CACC	0.189 (110)	0.196 (130)	−0.007	1.06 (1.00–1.13)
	CGCC	0.141 (82)	0.144 (95)	−0.003	1.05 (0.97–1.14)
	TGCC	0.086 (50)	0.064 (42)	**0.022****	0.77 (0.66–0.89)
	Rare haplotypes (<1%)	0.013 (8)	0.018 (12)	−0.004	1.35 (0.71–2.56)
Chr 19 *ERCC2*	**TGAA**	0.353 (205)	0.333 (219)	**0.021***	1
	CGCG	0.305 (117)	0.320 (211)	−0.015	1.11 (1.06–1.16)
	CCAG	0.104 (61)	0.109 (72)	−0.004	1.10 (0.99–1.22)
	CCCG	0.079 (46)	0.082 (54)	−0.003	1.10 (0.96–1.25)
	CGAA	0.053 (31)	0.053 (35)	0.000	1.06 (0.88–1.29)
	CGAG	0.037 (22)	0.030 (19)	0.007	0.85 (0.63–1.15)
	TGAG	0.025 (14)	0.025 (17)	0.000	1.07 (0.72–1.67)
	CCAA	0.023 (13)	0.024 (16)	−0.001	1.10 (0.72–1.67)
	TGCG	0.018 (10)	0.019 (13)	−0.002	1.17 (0.69–1.99)
	Rare haplotypes (<1%)	0.004 (2)	0.006 (4)	−0.002	1.76 (0.23–13.18)
Chr 19 *ERCC1*	**GAT**	0.571 (332)	0.606 (401)	**−0.035****	1
	GGC	0.240 (140)	0.253 (167)	−0.013	0.99 (0.94–1.04)
	AAC	0.170 (99)	0.120 (79)	**0.050****	0.67 (0.58–0.79)
	GAC	0.013 (7)	0.016 (10)	−0.003	1.15 (0.58–2.29)
	Rare haplotypes (<1%)	0.005 (3)	0.005 (3)	0.001	0.83 (0.13–5.40)

* Statistically significant at 95%.
** Statistically significant at 99%.

the first study and/or HWE deviating studies were excluded. On the other hand, two other DSBR genes, *NBN* and *XRCC4*, were still significantly associated after exclusion of the first study and/or HWE deviating studies, showing that DNA repair polymorphisms can still have an important role in bladder carcinogenesis even though no significant results have been reported about their association from the published bladder cancer GWA study [16], possibly due to the stringent genome-wide significance thresholds, or to unknown gene–gene and gene–gene–environment interactions not yet investigated.

In our study, after FDR correction we reported significant associations at single SNP analysis only for three *ERCC1* polymorphisms (rs967591, rs735482, rs2336219), whose rare alleles resulted to be protective against bladder cancer. The haplotype analysis in *ERCC1* gene further confirmed single SNP analysis with the GAT haplotype significantly more present in cases and AAC haplotype in controls. Many studies have been published on the possible association between *ERCC1* polymorphisms/haplotypes and cancer (http://www.episat.org/episat/huge/), or survival [22,23]. Also bladder cancer survival has been recently reported to be linked to ERCC1 polymorphisms [24]. However, the most important confirmation of the possible *ERCC1* polymorphisms/haplotypes involvement is due to functional studies relating *ERCC1* genetic variations DNA and repair capacity [25] or gene expression levels [26]. All these results strongly support a plausible role of *ERCC1* in cancer risk.

ERCC1 protein forms a heterodimer with XPF (also called ERCC4) to form the endonuclease which makes the 5′ incision during nucleotide excision repair.

The *ERCC1* gene consists of 10 exons spread over approximately 14 kb [27]. Another DNA repair gene, *XRCC1* (194360; OMIM), is located in this region [28]. In the course of characterizing *ERCC1*, Hoeijmakers et al. [29] found that its 3′ terminus overlapped with the 3′ end of another gene, designated *ASE1*. This exceptional type of gene overlap was conserved in the mouse and even in the yeast *ERCC1* homolog, *RAD10*, suggesting an important biologic function of this region (19q13.2–q13.3) and many reports have been published on the possible association of haplotypes in this region with increased cancer risk [30–33].

Further significant associations with other DNA repair genes have been found performing the haplotype analysis considering only tagSNPs to avoid redundancy of information: the frequency of *XRCC3* TAGT haplotype is significantly higher in cases than in controls; bearing the T allele at the SNP rs861539 has been reported to confer increased risk in different cancer studies [6], and specifically in breast and stomach cancers as reported in a recent meta-analysis [21].

XRCC3 participates in DNA double-strand break and cross-link repair through homologous recombination and contributes, as other RAD51-related proteins, to the maintenance of chromosomal stability [34–36]. The Thr241Met substitution in XRCC3 is a non-conservative change with possible biological implications for the functionality of the enzyme and/or for the interaction with other proteins involved in DNA repair.

Few years ago we also reported [5,37] on the association between bulky DNA-adduct formation and XRCC3 cod 241 (rs861539) Thr/Met and Met/Met genotypes (particularly in the slow NAT-2 group) that could be related to environmental exposure to genotoxic aromatic amines, such as *trans*-4-dimethylaminostilbene and 4-*trans*-acetylaminostilbene [38], which are capable of forming DNA adducts to guanine and adenine and of inducing other secondary lesions of equal or greater importance, e.g., cross-links between bases. 4-Aminostilbene has been reported to induce high levels of chromosomal aberrations [39]. The association between DNA adducts and the *XRCC3* polymorphism may also be due to oxidation reactions, which might cause formation of intrastrand cross-links between adjacent nucleotides, leading to bulky oxidative DNA modification, i.e., dimer formation, detectable by 32P-DNA post-labeling [40].

Along these lines, *XRCC3* involvement could be plausible because it takes part in the repair process of cross-links and DSBs which seem very frequent in bladder cancer cells leading to high levels of chromosomal rearrangements.

We also found a highly significant difference for *MGMT*-TA haplotype and for *XRCC1*-TGCC haplotype and a significant difference for *ERCC2*-TGAA haplotype, all more represented in controls.

Conflicting results have been reported on the associations between some SNPs in all the above mentioned genes and different types of cancer (http://www.episat.org/episat/huge/): the haplotype analyses could possibly help to further clarify their involvement in cancer risk.

Only one study has investigated the relationship among *MGMT* haplotypes and bladder cancer [41] and some others analysed the relationship with other cancer sites [42].

MGMT is a protein involved in the cellular defense against the biological effects of O6-methylguanine (O6-MeG) in DNA. It repairs alkylated guanine in DNA by stoichiometrically transferring the alkyl group at the O6 position to a cysteine residue in the enzyme. This is a suicide reaction: the enzyme is irreversibly inactivated.

Our results, together with other published studies on very large case–control studies [43,44], seem to suggest that genes belonging to different DNA repair pathways could be involved in increasing bladder cancer risk.

The NER system involvement in bladder cancer has been recently investigated by Garcia-Closas et al. [43] in a large case–control study by analyzing 22 SNPs in seven NER genes. They found a small increase in bladder cancer risk in subjects carrying variant alleles compared to subjects with the homozygous wild-type genotypes for RAD23B IVS5-15A>G, ERCC2 R156R, ERCC1 IVS5+33A>C and ERCC5 M254V. A global test for pathway effects indicated that genetic variation in NER characterized by the 22 SNPs analysed in this study significantly predicts bladder cancer risk.

Similarly, high-order interactions among genetic polymorphisms in NER pathway genes and smoking in modulating bladder cancer risk have been reported [45]. In multifactor dimensionality reduction (MDR) analysis, the five-factor model including smoking, *CCNH* V270A, *ERCC6* M1097V, *RAD23B* A249V and *XPD* D312N had the best ability to predict bladder cancer risk. The contributions of these polymorphisms may jointly affect bladder cancer risk through gene–gene and gene–smoking interactions.

Moreover, the same group (Figueroa et al. [44]) reported about 39 SNPs in seven candidate genes whose products are involved in DSBR pathway. They found that the genetic variants investigated significantly contributed to bladder cancer risk (global likelihood ratio test $p = 0.01$), in particular considering polymorphisms in *ZNF350, XRCC4* and *XRCC2* genes.

Our limited analysis of epistasis by using MDR, showed that the best model suggests an interaction between the rs2336219 SNP of *ERCC1* gene and the rs1799782 SNP of *XRCC1* gene, whereas the dendrogram suggests a synergic relationship between these SNPs, with the joint effect of these two SNPs being correlated with the rs1799796 SNP of *XRCC3* gene.

In a collaborative study [46], we recently investigated interactions between polymorphisms in *APE1*, *XRCC1*, *XRCC1*, *XPD*, *XPC* and *XRCC3* by using four analytic approaches: logistic regression, Multifactor Dimensionality Reduction (MDR), hierarchical interaction graphs, classification and regression trees (CART), and logic regression analyses. All five methods supported a gene–gene interaction between XRCC1-399/XRCC3-241; three methods predicted an interaction between XRCC1-399/XPD-751. On the other hand, we did not find any interaction between fruits and vegetables consumption and DNA repair gene polymorphisms [47].

The present study has some limitations. We have controlled our analyses using self-reported smoking and food-intake data, but self-reported data may have poor quality, particularly for diet in a case–control setting. Also selection bias can arise from a hospital-based case/control study. However, we tested genetic instead of environmental hypotheses, so these problems were reduced. It could be useful to replicate these results in other studies. Recent genome-wide association studies on bladder cancer [16,48] did not show highly statistically significant associations for any of the above analysed common DNA repair gene variants. Therefore, even if some of the DNA repair genes are associated with bladder cancer, the signals observed in GWA studies would not necessarily be among the reported low-lying fruit (i.e. the polymorphisms with the lowest *p*-Values).

In conclusion, our findings provide support for the influence of genetic variation in DNA repair genes on bladder cancer risk and suggest that the effects of high-order interactions should be taken into account as modulating factors affecting bladder cancer risk. A detailed characterization of DNA repair genetic variation is warranted and might ultimately help to identify multiple susceptibility variants that could be responsible for joint effects on the risk.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2009.12.002.

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