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Short Report

Polymorphisms in the *XRCC1* gene modify survival of bladder cancer patients treated with chemotherapy.

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Only few studies investigated the role of DNA repair genetic variants in survival for bladder cancer. In this study on 456 bladder cancer patients it has been observed that chemotherapy treated cancer patients bearing a higher number of genetic variants in *XRCC1* gene had a better survival, suggesting that a proficient DNA repair may result in resistance to therapy and shorter survival. This finding may have clinical implications for the choice of therapy.

Abstract

Survival of bladder cancer patients depends on several factors including disease stage and grade at diagnosis, age, health status of the patient and the applied treatment. Several studies investigated the role of DNA repair genetic variants in cancer susceptibility, but only few studies investigated their role in survival and response to chemotherapy for bladder cancer.

We genotyped 28 single nucleotide polymorphisms (SNP) in DNA repair genes in 456 bladder cancer patients, reconstructed haplotypes and calculated a score for combinations of the SNPs. We estimated Hazard Ratios (adjHR) for time to death.

Among patients treated with chemotherapy, variant alleles of five SNPs in the *XRCC1* gene conferred better survival (rs915927 adjHR 0.55 (95%CI 0.32-0.94); rs76507 adjHR 0.48 (95%CI 0.27-0.84); rs2854501 adjHR 0.25 (95%CI 0.12-0.52); rs2854509 adjHR 0.21 (95%CI 0.09-0.46); rs3213255 adjHR 0.46 (95%CI 0.26-0.80). In this group of patients, an increasing number of variant alleles in a *XRCC1* gene score was associated with a better survival (26% decrease of risk of death for each additional variant allele in *XRCC1*). By functional analyses we demonstrated that the previous *XRCC1* SNPs confer lower DNA repair capacity. This may support the hypothesis that survival in this patients may be modulated by the different DNA repair capacity determined by genetic variants. Chemotherapy treated cancer patients bearing an increasing number of “risky” alleles in *XRCC1* gene had a better survival, suggesting

that a proficient DNA repair may result in resistance to therapy and shorter survival.

This finding may have clinical implications for the choice of therapy.

Key words

Bladder cancer; Chemotherapy; DNA repair genes; Survival; *XRCC1*.

1. Introduction

Survival of bladder cancer patients varies widely, depending on the tumor stage and grade at diagnosis, age, general health of the patient and the applied treatment ¹. In Europe, the age-standardised 5-year survival of all bladder cancer patients is 72.8% in men and 69.3% in women ². In Italy, survival is 77.0% for both sexes ². More than 80% of bladder cancers are non-invasive (Ta/T1) at diagnosis and have a good prognosis in response to transurethral resection (TURB) followed by intravesical chemoprophylaxis (e.g. Gemcitabine, Mitomycin C, Epirubicine) or immunoprophylaxis (Bacillus Calmette Guerin, BCG)) ³. Only one third of T1 patients that do not respond to these treatments, experience progression to a higher stage / grade recurrence of a tumour with a poor prognosis ⁴. In case of recurrent pT1 G3 or carcinoma *in situ* (CIS) and poor response to intravesical chemotherapy or BCG, the following therapy is cystectomy ⁵. The tumours that are muscle-invasive at diagnosis (T2+) have a poor prognosis. Therapy for high grade muscle invasive tumours is radical cystectomy. Adjuvant chemotherapy for patients with pT3/pT4 and/or N+ disease is under debate ⁶. Radiotherapy is used in selected cases of muscle invasive cancers to allow organ preservation.

Although the major prognostic determinants for bladder cancer are tumor stage and grade at diagnosis, differences in survival time among patients with similar disease stage and treatment remain unexplained.

Until now, genetic variants (such as single nucleotide polymorphisms, SNPs) have been extensively studied in relation to cancer risk ⁷. Few studies have been conducted on the relationship between SNPs and inter-individual survival variability in response to chemotherapy for bladder cancer ⁸⁻¹⁰. Also, little is known about the role of DNA repair gene polymorphisms in survival of bladder cancer ^{8, 9, 11, 12}, although there is evidence of their involvement in other tumour sites, such as the lung ¹³.

In the present study we investigate the association between 28 SNPs in 8 different DNA repair genes and survival from bladder cancer.

2. Subjects and methods

2.1 Subjects

The study population included all newly diagnosed, histologically confirmed cases of bladder cancer registered at two urology departments of S. Giovanni Battista hospital in Turin (Italy) during the years 1994-2008¹⁴. All subjects were men, aged 40–75 years and living in the Turin metropolitan area. Before any treatment, a trained interviewer used a detailed questionnaire to conduct a face-to-face interview. All subjects in the study signed an informed consent form. The type of therapy (e.g., BCG, chemotherapy, radiotherapy) was recorded through the perusal of clinical records, in collaboration with urologists. Patients treated with one instillation of chemotherapy immediately after TUR were not considered in the chemotherapy group.

Vital status was ascertained through linkage with the local demographic office and death certificates were retrieved to identify the specific causes of death (last follow up 31/03/2012).

2.2 DNA extraction and SNP genotyping

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) before therapy and stored at -20 °C until DNA extraction. 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 samples and by a standard salting-out technique for the rest.

We selected 28 SNPs in 8 different DNA repair genes based on the following criteria: i) variants previously associated in the literature with bladder cancer risk ii) variants that show a biological significance either because they induce an aminoacidic substitution in the corresponding protein or they were associated with alterations in the enzymatic function.

We genotyped all the polymorphisms using a 5' Nuclease assay (TaqMan) with fluorogenic Minor Groove Binder probes (for genotyping method description ¹⁵).

2.3 DNA typing quality control

Technical validation included a comparison between genotypes obtained by PCR-Restriction Fragment Length Polymorphism, Denaturing High Performance Liquid Chromatography and Taq-Man assays: at least 10% of the genotyping was randomly repeated for each polymorphism. Concordance was between 99-100% for all the comparisons; discordant genotypes were excluded from the analysis. We also fully repeated the genotyping of two polymorphisms with a concordance of 98% for rs861531 and 100% for rs26651 .

2.4 Statistical analysis

The characteristics of the patients are described as absolute and relative frequencies for qualitative variables, and means and standard deviations for quantitative variables. We used chi-square tests or the Wilcoxon Rank Test, to assess statistical significance.

For each SNP we calculated crude and adjusted (by age, smoking status, stage and grade) per allele Hazard Ratios (HR) and the corresponding 95% Confidence Intervals (95% CI) using the Cox proportional hazards regression model. Analyses were stratified by therapy (intravesical or systemic chemotherapy *versus* BCG alone or no medical treatment) and performed according to co-dominant and per-allele models. Subjects with missing values for adjustment variables were removed.

We reconstructed haplotypes using Linkage Disequilibrium (LD) and 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 with a Markov Chain Monte Carlo method. For each haplotype we computed the HR, comparing subjects carrying 2, 1 versus 0 copies of the same haplotype. The following SNPs in *XRCC1* gene had an LD value of $R^2 > 0.80$: rs762507 - rs3213255 ($R^2 = 0.83$) and rs915927 – rs2854501 ($R^2 = 0.86$). The analyses of LD were performed with Haploview 4.1. The phase of haplotypes was inferred using PHASE 2.1.

All the analyses were performed using SAS v 9.2 and STATA v 10.0.

3. Results

We recruited 456 male bladder cancer patients, 139 of whom died of cancer during the observation period. The mean age at diagnosis was 63.38 years (SD 7.64) and the median follow up time was 7.85 years.

Among patients with known stage ($N=449$), 387 (86.2%) had a non-muscle invasive cancer, while 62 (13.8%) had a stage 2 tumour or greater at diagnosis. Differences in survival by stage and grade were statistically significant (P -value for log-rank test <0.0001 for both predictors). Almost thirty percent of patients were treated with intravesical and/or systemic chemotherapy, while the rest of patients was treated with BCG or did not receive any medical treatment (Table 1).

In Table 2 we show the association between variant alleles for the DNA repair genes and overall survival, stratified by type of treatment (HR estimated using per-allele models). Variant alleles of five different polymorphisms (rs915927, rs762507, rs2854501, rs2854509, rs3213255) in the *XRCC1* gene confer a longer survival, for patients treated with chemotherapy in both the crude and adjusted models. In addition, in those patients treated with chemotherapy a SNP in the *ERCC2* gene (rs1711140) conferred a shorter survival, in both crude and adjusted models.

Results from haplotype analysis confirmed single SNP analysis, showing an increased survival in chemotherapy treated patients carrying the GGCTA haplotype in *XRCC1* polymorphisms with an HR of 0.35 (95% CI 0.18-0.69) (data not shown).

The analysis for the sum of variant alleles in the *XRCC1* gene showed a strong increasing trend in survival, only for patients treated with chemotherapy (Table 3). The risk of death decreased by 26% for each additional variant allele.

4. Discussion

In the present study it has been observed that patients who received chemotherapy, with a higher number of genetic variants in the *XRCC1* base-excision repair gene (BER), had a significantly reduced risk of death (26% for each additional variant allele). In contrast, the variant allele for a SNP in the *ERCC2* nucleotide excision repair (NER) gene confers a shorter survival. This finding may have important clinical implications for predicting the patients who will respond to chemotherapy. Previous studies on bladder cancer have suggested that genetic polymorphisms can influence prognosis, perhaps by modulating the metabolism of chemotherapeutic agents or by affecting the repair of DNA adducts formed by drugs such as cisplatin¹⁶.

Recently, the role of DNA repair gene polymorphisms in bladder cancer survival has received increasing attention. In particular, Sakano *et al.*¹⁷ found that combined genotypes with at least one variant allele in the *ERCC2* or *XRCC1* genes were significantly associated with improved cancer-specific survival in a group of 78 muscle-invasive bladder cancer patients treated with platinum based systemic chemotherapy. The same group published a study on 101 bladder cancer patients in which they found that the presence of more than two variant alleles in NER genes, including *XPC*, *ERCC2* and *XPG*, was significantly associated with acute toxicity of platinum based chemotherapy for bladder cancer. Additionally, Sanyal *et al.*¹⁸ found that bladder cancer patients carrying the variant allele of an *ERCC2* polymorphism had an increased survival when compared with non-carriers. Similarly, the presence of variant alleles from

ERCC2 (rs1052559) and *XPC* (rs2228001) polymorphisms in combination, showed an increasing in survival. Finally, carriers of the variant allele for *XRCC1* rs25487 polymorphism showed a lower risk of death than the non-carriers after instillation with BCG or Mitomycin C and radiotherapy ¹⁸.

In general, for bladder cancer the majority of the studies investigating the role of DNA repair SNPs in survival focused their analysis on small groups of patients treated with platinum-based systemic chemotherapy and/or radiotherapy. In bladder cancer patients similar treatments are usually confined to muscle-invasive and drug-resistant cancers. However, the majority of bladder cancers in Western countries are non-muscle invasive. In the current clinical practice the medical treatment of choice for superficial bladder cancer has become intravesical instillation of chemotherapeutic agents such as Gemcitabine, Mitomycine C and Epirubicine ⁵.

Cancer therapy involves the exposure of the body to agents that kill cancer cells more efficiently than normal tissue cells. Cancer cells proliferate more rapidly than their normal counterparts so most cancer drugs target the cell cycle and one of the most common means of targeting cell cycle is to exploit the effect of DNA-damaging drugs. The efficacy of DNA damage-based cancer therapy can thus be modulated by DNA repair pathways. In agreement with some studies on lung cancer patients treated with cisplatin ¹³, the response to chemotherapy may depend upon the ability of drugs to damage DNA. Such damage, if left un-repaired, can lead to cell death and hence to better response to therapy. DNA repair pathways protect cells against DNA damage–

induced apoptosis and genomic instability. Thus, a proficient DNA repair capacity can result in cellular resistance to therapy and consequently in shorter survival. These features make DNA repair mechanisms and its modulation due to the presence of SNPs a promising field of research for the evaluation of the efficacy of anticancer treatments.

In the present study we investigated survival in patients treated with different therapeutic agents. For patients treated with chemotherapy (local and systemic), we found that variant alleles of five different polymorphisms in the *XRCC1* gene confer a longer survival and a single SNP in *ERCC2* gene confers a shorter survival. These results, in particular for *XRCC1* SNPs, are confirmed by the haplotype analysis and by a dose-response analysis of the total number of variant alleles. We can hypothesize that carriers of one or more variant alleles of the *XRCC1* gene may confer a lower DNA repair capacity and consequently a better response to therapy.

XRCC1 encodes for a protein involved in the BER pathway and, more specifically, in the repair of single-strand breaks. It is a scaffold for other DNA repair proteins, such as DNA polymerase β 4 and DNA ligase III. The BER pathway can be involved in repairing DNA crosslinks induced by Epirubicine ¹⁹ and the DNA damage induced by alkylating agents such as Mitomycin C ²⁰. There have been a number of experimental efforts to characterize biochemical activities of *XRCC1* according to its variants: only rs25489 displayed a mild defect in DNA binding capacity and some differences in the protein activity, perhaps underlying a repair deficiency ²¹. The disparity in experimental results and the lack of evidence for the other SNPs may stem

from the fact that those variants impart only a minor defect on protein function maybe difficult to detect.

ERCC2 encodes for a protein involved in the NER pathway that can remove DNA cross links. NER substrates include bulky chemical adducts, DNA intra-strand crosslinks and some forms of oxidative damage. We can hypothesize that carriers of the variant allele of *ERCC2* rs171140 have a better DNA repair capacity and therefore a worse response to therapy. The NER pathway can be involved in the repair of DNA damage by Epirubicine and Gemcitabine.

The results of another large study from our research group on healthy subjects ²² support the hypothesis of an effect of SNPs in the modulation of DNA repair capacity. DNA repair capacity was evaluated in different functional assays on peripheral blood mononuclear cells (PBMCs) from 120 young healthy subjects: γ H2AX phosphorylation assay to measure double-strand break repair (DSBR) ²², an aphidicolin-block comet assay to measure NER capacity ²³ and an 8-oxoguanine DNA glycosylase (OGG1) activity assay to measure BER activity ²⁴. A genotype-phenotype association analysis between 768 SNPs in DNA repair genes and cellular DNA repair capacity was performed. For the *XRCC1* and *ERCC2* genes, we found that 4 out of the 5 *XRCC1* SNP variants (rs915927, rs2854509, rs2854501, rs3213255) that were associated in this study with survival, conferred a lower DNA repair capacity in NER and DSBR pathways, while the *ERCC2* SNP variant rs1618536 (LD with rs171140: $R^2=0.99$) conferred an increased NER and DSBR capacity (data not shown). Moreover, from an *in silico* analysis by using functional prediction software (Pupasuite, SNPs3D and

others) *XRCC1* rs915927 and *ERCC2* rs171140 SNPs were found to reside within putative exon splicing enhancers.

The analysis of two out of six SNPs (rs2854501, rs3213255) was replicated in a case control study who recruited 868 histologically confirmed incident bladder cancer patients at the MD Anderson Cancer Center in Texas (US) ²⁵. However, in this US independent study, although the risk effects were in the same direction of our study, we were not able to replicate the association of two *XRCC1* SNPs and survival from bladder cancer (in crude model, rs2854501 HR 0.88 95%CI 0.64-1.23 and rs3213255 HR 0.87 95%CI 0.66-1.15; in fully adjusted model, rs2854501 HR 0.97 95%CI 0.68-1.38 and rs3213255 HR 1.03 95%CI 0.77-1.38). These discordant results may be due to a fundamental genetic heterogeneity between the South European and US populations as supported by the I^2 heterogeneity values (I^2 rs2854501 =86.9% $p<0.006$ and I^2 rs3213255 =85.7% $p<0.008$). This feature, in combination with the application of different therapeutic schemes, may contribute to differences in survival conferred by the identified SNPs. Therefore, the association found in the Turin study needs replication in other European samples to verified if this SNP contribute to survival for bladder cancer in chemotherapy treated patients.

Finally, although there are only few studies investigating the role of DNA repair genetic variants in survival and response to chemotherapy for bladder cancer, we are aware of the possible limitation of our study due to the small sample size when stratifying for chemotherapy treatment.

In conclusion, we observed an association between DNA repair polymorphisms and survival. These data were supported by our previous functional studies, which suggested potential mechanisms. DNA repair genotype profiles may therefore be a way to personalize therapeutic schemes. The consistency of the observed effects on survival for 5 SNPs in the same gene (*XRCC1*), and the dose-response effect favour the hypothesis that these variants specifically modulate the survival of chemotherapy treated patients.

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Table 1. Distribution of patients (N=456) by clinical and demographic characteristics.

Variables	Deceased of cancer N (%)	Alive N (%)	P
	N = 139	N = 317	
Age (years)			
Mean (SD)	66.8 (6.6)	62.0 (7.6)	<0.001
Smoking Status			
Current	65 (46.8)	149 (47.1)	0.24
Former	70 (50.4)	146 (46.2)	
Never	4 (2.8)	21 (6.7)	
Tumour stage			
pTa/T1	101 (72.7)	286(92.3)	<0.001
pT2+	38 (26.3)	24 (7.7)	
Tumour grade			
G1	28 (20.3)	112 (27.6)	<0.001
G2	47 (34.1)	116 (39.8)	
G3	63 (45.6)	87 (35.6)	
Treatment			
Only BCG or no medical treatment	94 (67.6)	208 (65.8)	0.74
Intravesical chemotherapy	38 (27.3)	108 (34.1)	
Systemic chemotherapy	7 (5.1)	0 (0.0)	
Radioterapy	21 (15.1)	10 (3.2)	<0.001
Radical cystectomy	43 (30.9)	44 (13.9)	<0.001

Table 2. Associations between SNPs and survival, stratified by medical treatment. * Adjusted for age, smoking status, stage and grading.

Chromosomal band	Gene	Repair pathway	rs	Alleles	No chemotherapy				P-value	Chemotherapy			
					N	crude HR	adjusted HR*	P-value		N	crude HR	adjusted HR*	P-value
3p25	XPC	NER	2228001	A>C	203	1.09 (0.72-1.65)	0.94 (0.62-1.43)	0.65	124	0.90 (0.54-1.50)	1.22 (0.71-2.09)	0.92	
9q22.3	XPA	NER	1800975	G>A	199	1.09 (0.71-1.67)	0.94 (0.65-1.67)	0.47	118	0.52 (0.34-0.90)	0.74 (0.41-1.33)	0.32	
10q26	MGMT	Direct reversal	12917	C>T	218	0.83 (0.45-1.54)	0.97 (0.51-1.87)	0.94	129	1.36 (0.73-2.54)	1.28 (0.67-2.43)	0.45	
14q11.2	APEX	BER	1130409	T>G	216	1.33 (0.92-1.92)	1.52 (0.99-2.23)	0.06	128	0.97 (0.63-1.48)	1.09 (0.65-1.42)	0.13	
14q32.3	XRCC3	DSBR/HR	861539	C>T	293	0.92 (0.68-1.22)	0.93 (0.68-1.26)	0.64	152	0.88 (0.56-1.39)	0.95 (0.60-1.50)	0.82	
			1799796	A>G	288	1.36 (0.96-1.87)	1.06 (0.76-1.49)	0.70	148	0.97 (0.59-1.59)	1.19 (0.72-2.90)	0.49	
			861531	G>T	203	0.98 (0.66-1.46)	0.91 (0.60-1.39)	0.67	123	0.85 (0.51-1.44)	1.09 (0.61-1.91)	0.77	
			861530	G>A	203	0.80 (0.52-1.26)	0.95 (0.60-1.52)	0.86	123	1.31 (0.77-2.23)	1.02 (0.59-1.75)	0.94	
19q13.2	XRCC1	BER	25487	G>A	294	0.17 (0.87-1.37)	1.06 (0.78-1.46)	0.62	147	1.27 (0.82-1.96)	1.42 (0.91-2.24)	0.12	
			915927	A>G	215	0.81 (0.17-1.14)	0.82 (0.57-1.17)	0.27	114	0.78 (0.48-1.26)	0.55 (0.32-0.94)	0.03	
			1799782	C>T	294	0.62 (0.81-1.42)	0.86 (0.43-1.71)	0.66	149	0.62 (0.22-1.75)	0.55 (0.18-1.65)	0.29	
			762507	G>A	205	0.65 (0.41-1.02)	0.74 (0.46-1.17)	0.20	123	0.83 (0.50-1.37)	0.48 (0.27-0.84)	0.01	
			1799778	C>A	204	1.20 (0.80-1.78)	1.16 (0.75-1.80)	0.48	123	1.37 (0.86-2.18)	1.76 (0.90-3.06)	0.07	
			2854501	C>T	191	0.69 (0.38-1.26)	0.82 (0.44-1.57)	0.56	120	0.48 (0.25-0.94)	0.25 (0.12-0.52)	0.001	
			1001581	C>T	199	0.21 (0.83-1.77)	1.05 (0.72-1.54)	0.77	122	1.36 (0.84-2.21)	1.96 (0.98-3.59)	0.06	
			2854509	C>A	205	0.78 (0.42-1.46)	0.78 (0.41-1.49)	0.47	122	0.43 (0.22-0.88)	0.21 (0.09-0.46)	0.001	
19q13.3	ERCC2	NER	3213255	T>C	200	0.80 (0.51-1.26)	0.94 (0.60-1.46)	0.79	125	0.73 (0.44-1.20)	0.46 (0.26-0.80)	0.01	
			13181	A>C	294	1.05 (0.79-1.41)	1.17 (0.85-1.63)	0.32	151	0.51 (0.38-0.98)	0.80 (0.48-1.31)	0.38	
			1052555	C>T	184	0.79 (0.49-1.28)	0.81 (0.49-1.36)	0.71	107	0.78 (0.44-1.37)	0.98 (0.56-2.06)	0.80	
			1799787	C>T	194	0.88 (0.57-1.37)	0.93 (0.58-1.47)	0.44	118	0.77 (0.46-1.28)	0.96 (0.57-1.96)	0.92	
			3916874	G>C	186	0.25 (0.76-2.06)	1.17 (0.68-2.01)	0.56	114	1.31 (0.77-2.28)	1.15 (0.68-1.84)	0.90	
			171140	A>C	198	1.03 (0.68-1.55)	1.07 (0.70-1.61)	0.75	121	1.71 (1.03-2.85)	2.07 (1.06-4.28)	0.03	
19q13.3	ERCC1	NER	1799793	G>A	281	1.20 (0.88-1.64)	1.16 (0.84-1.63)	0.36	146	0.77 (0.50-1.19)	0.96 (0.61-1.53)	0.89	
			967591	G>A	201	2.21 (0.70-2.08)	1.89 (0.95-2.22)	0.35	123	0.48 (0.20-1.16)	0.60 (0.24-1.45)	0.25	
			735482	A>C	203	1.22 (0.70-2.11)	1.29 (0.75-2.12)	0.43	120	0.54 (0.22-1.34)	0.64 (0.26-1.55)	0.32	
			2336219	G>A	201	1.22 (0.69-2.18)	1.25 (0.72-2.17)	0.41	121	0.51 (0.21-1.26)	0.62 (0.26-1.52)	0.29	
			3212955	A>G	201	1.47 (0.99-2.20)	1.18 (0.76-1.85)	0.45	120	0.78 (0.43-1.44)	0.78 (0.42-1.45)	0.44	
			11615	T>C	245	1.35 (0.98-1.91)	1.15 (0.81-1.62)	0.43	136	0.68 (0.43-1.10)	0.67 (0.43-1.07)	0.11	

Table 3. Distribution of patients and association between the sum of low-activity alleles in the *XRCC1* gene and survival (only chemotherapy treated patients (intravesical+systemic)).

	Deceased of cancer <i>N</i>	Alive <i>N</i>	HR crude (95% CI)	HR adjusted (95% CI)*
0 <i>XRCC1</i> variant alleles	9	15	Ref	Ref
1 <i>XRCC1</i> variant allele	8	18	0.71 (0.27-1.85)	0.39 (0.12-1.26)
2-4 <i>XRCC1</i> variant alleles	5	15	0.50 (0.17-1.50)	0.28 (0.08-1.18)
> 4 <i>XRCC1</i> variant alleles	3	16	0.35 (0.94-1.29)	0.05 (0.01-0.34)

*Adjusted for age, smoking status, stage and grading.