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ScienceDirect
EJSO 37 (2011) 727–733
EJSO the Journal of Cancer Surgery
www.ejso.com

Association of death receptor 4, Caspase 3 and 5 gene polymorphism with increased risk to bladder cancer in North Indians

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Accepted 23 May 2011
Available online 22 June 2011

Abstract

Purpose: Perturbed apoptosis due to missense alterations in candidate tumor suppressor gene Death receptor 4 (*DR4*) and in caspases (*Casp*) lead to deregulated cell proliferation and cancer predisposition. Some data indicate that normal variations within the sequence of apoptotic genes may lead to suboptimal apoptotic capacity and therefore increased cancer risk. To test our proposal we examined whether six single nucleotide polymorphisms (SNPs) of the *DR4* and *Casp3, 5* genes contrive the risk of bladder cancer (BC) in a North Indian population.

Materials and methods: Genotyping was performed in 200 BC patients and 225 controls by Allele-specific PCR and by polymerase chain reaction-restriction fragment length polymorphism.

Results: In *DR4 Arg141His,* BC patients having AA genotype ($p = 0.036$; OR = 2.51. In *Casp5Leu13Phe* G > C, significant association was observed with GC ($p = 0.025$; OR = 1.78) and also in GC + CC ($p = 0.026$; OR = 1.68). C allele carriers in *Casp5Ala90Thr* T > C showed low risk of BC ($p = 0.036$; OR = 0.83). While in *Casp3* G > A, AG ($p = 0.003$; OR = 2.11), GG ($p = 0.050$; OR = 2.18), G allele ($p < 0.001$; OR = 1.85) and its carrier AG + GG ($p = 0.001$; OR = 2.12) have shown significant BC risk. Significant association between *DR4 Ala228Glu* polymorphism and smoking was observed in BC risk. Haplotype analysis demonstrated that *DR4* (*Thr209Arg–Arg141His–Ala228Glu*) C-G-C is associated with 1.8 folds (OR = 1.85; $p = 0.033$) risk. GG genotype of *Casp3* G > A polymorphism showed increased risk of recurrence ($p = 0.009$; HR = 5.20).

Conclusion: This study provided new support for the association of *DR4* and *Casp3, 5* in BC development, the tumorigenic effect of which was observed to be more enhanced in case of smoking exposure.

Keywords: Apoptosis; *Bacillus* Calmette-Guerin; Bladder cancer; Haplotype; Polymorphism

Introduction

Apoptosis plays a role in the elimination of DNA-damaged cells thus protecting the host from cancer development. An imbalance between cell death and proliferation may result in tumor formation. Some data indicate that normal variations within the sequence of apoptotic genes (such as death receptors and caspases, etc) may lead to suboptimal apoptotic capacity and therefore increased cancer risk. The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) activates the extrinsic apoptotic pathway through the engagement of the proapoptotic death receptor 4 (DR4, TNFRSF10A, TRAILR-1), a member of the tumor necrosis factor receptor super family. *DR4* consists of two extracellular cysteine-rich, ligand-binding pseudo-repeats (50s and 90s loops), one single transmembrane helix as well as the apoptosis-triggering cytoplasmic death domain. Suppression of cell death signaling due to detrimental alterations in *DR4* involves a deregulated cell proliferation and predisposes to cancer.[1–6] Previous studies have shown that *DR4* and caspases are associated with bladder cancer (BC) and are able to predict stage, grade, and disease outcome.[7,8] A recent study also demonstrated that genetic variants of the *DR4* gene may be involved in

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doi:10.1016/j.ejso.2011.05.013

the etiology of BC in Chinese population.[9] *DR4* mutations have been described in different human cancers, such as lung, head and neck cancer, non-Hodgkin's lymphoma as well as in breast cancer and breast cancer cell lines[10–12] which points to *DR4* as an attractive candidate tumor suppressor gene.

Caspases are a family of highly conserved intracellular aspartate-specific cysteine proteases that are key intermediaries of the apoptotic process. Various molecular epidemiological studies suggest that single nucleotide polymorphisms (SNPs) may contribute to individual susceptibility to BC by affecting either the expression or the activities of various enzymes.[3,13] However, the role of selected SNPs in major apoptosis-regulatory caspase genes in bladder cancer (BC) remains to be explored.

As the genetic nature of BC is complex, individual polymorphisms are likely to have a modest effect on risk. It is also plausible that examining several polymorphisms within biologically relevant pathways may reveal subgroups of individuals who are at significantly elevated risk for this disease. Hence we hypothesized that these genes and their haplotypes involved in the initiation of apoptosis may be susceptible to BC risk. In this study, we identified the possible association of *DR4 Thr209Arg C > G, Arg141His G > A, Ala228Glu C > A* and *Casp5Leu13Phe G > C, Ala90Thr T > C and Casp3 G > A* genes in BC patients and healthy controls to assess their association with the overall risk of BC and the risks for superficial and invasive disease.

Materials and methods

Study subjects

The BC patients in this analysis were enrolled from an on-going case-control study of BC, which started patient recruitment in 2004. All enrolled patients were histologically confirmed invasive or superficial bladder cancer and were recruited from the department of Urology at Sanjay Gandhi Postgraduate Institute of Medical Sciences, a tertiary care center, from May 2004 to June 2009. A total of 200 BC patients (mean age 58.5 years; 175 men and 25 women) were employed for the study. Those with previous history of other cancer, cancer metastasized to the bladder from another origin, were excluded. Healthy and genetically unrelated individuals visiting the hospital for a routine checkup or health awareness camps and hospital employees were recruited as the controls ($n = 225$). All the controls were age and sex matched with similar ethnicity and had no evidence of malignancy or chronic disease. The mean age of the controls was 56.8 years, and M:F ratio as 201:24. The participation rate was 100%, and blood samples were available for all subjects. An epidemiologic questionnaire was designed for study participants to collect data on demographic characteristics, smoking history, occupation history, and other lifestyle factors were accounted. We stratified the patients into non-smokers (never smoke) and smokers (smoke more than 5 years). At the end of the interview, a 5-ml blood sample was drawn into coded tubes. Informed and written consent was taken from all subjects when interviewing for the demographic details and blood sample collection. The Ethical Review Board of the Institute approved the study.

Clinical data collection

The demographic and clinical characteristics of the patients are presented in Table 1. The clinical information about tumor size, number, stage and tumor grade, intravesical therapy and dates of recurrence, chemotherapy, radical cystectomy and pathological findings at cystectomy were provided by the urologists in our department. The tumor stages were classified as per American Joint Committee

Table 1
Demographical details of urinary bladder cancer patients and healthy controls.

Variable	Cases (200) *N* (%)	Controls (225) *n* (%)	Chi square-value
Sex			
Female	25(12.5)	24(10.5)	0.531
Male	175(87.5)	201(89.5)	
Age (Years)			
Mean age±SD	58.5 ± 12.4	56. 8 ± 10.8	0.117[a]
Median	58.0	57.0	
Smoking[b]			
Never Smokers	47(30.1)	174(77.3)	<0.001
Smokers	109(69.9)	51(22.7)	
Clinical Features			
Tumor number[b]			
Single	115(60.8)	–	–
Multiple	74(39.2)	–	
Tumor Size (cm)[b]			
<1	35(24.3)	–	–
1–3	73(50.7)	–	
>3	36(25.0)	–	
Stage			
Ta	64(32.0)	–	–
T1	85(42.5)	–	
T2	51(25.5)	–	
Grade			
G1	67(33.5)	–	–
G2	43(21.5)	–	
G3	90(45.0)	–	
Intravesical Therapy			
Non-treated	71(47.7)	–	–
BCG Induction (BCG i + m)	78(52.3)	–	
Event			
Recurrence	65(43.9)	–	–
Non-Recurrence	83(56.1)	–	

[a] Student *t*-test was used to determine the *p*-value.
[b] The sum could not add up to the total due to some missing values.

on Cancer's TNM staging system.[14] Of the 200 total patients enrolled in the study, 149 patients had non muscle invasive bladder cancer (NMIBC) while the rest 51 had muscle invasive bladder cancer (MIBC). Patients with NMIBC at high risk (high grade, multiple and large tumor) were treated with intravesical *Bacillus* Calmette-Guerin (BCG) ($n = 78$). The patients with NMI cancer of low risk (low grade and single small tumor) were kept on cystoscopic surveillance and considered as Non BCG patients. Subsequently, all the patients were examined by cystoscopy after every 3 months in first and second years and later at six months interval as long as there was no tumor recurrence. BCG treatment consisted of 6 weekly instillation induction BCG ($n = 78$). Since the number of patients receiving maintenance BCG was too low, we did not categorize the patients according to BCG regime for statistical analysis. The end point of study included tumor recurrence, defined as a newly found bladder tumor following a previous negative follow-up cystoscopy, or end of study time (60 months). Patients with invasive BC ($n = 51$) were treated with radical cystectomy with or without adjuvant chemotherapy, which included cisplatin, gemcitabine followed by periodical cystoscopy.

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by salting out method.[15] Polymorphisms in *DR4 Thr209Arg C > G, Arg141His G > A, Ala228Glu C > A* and *Casp5Leu13Phe G > C, Ala90Thr T > C and Casp3 G > A* were analyzed using AS-PCR (Allele-specific Polymerase Chain Reaction) and RFLP (Restriction Fragment Length Polymorphism). Details of the primers and cycle conditions for *DR4 and Casp3, 5* were derived from earlier published work.[8,16] Positive and negative controls were used in each genotyping assay, and 5% of the samples were randomly selected and run in duplicates with 100% concordance. The results were reproducible with no discrepancy in genotyping.

Statistical analysis

The power of the study was calculated using Quanto software, version 1.0 (available from: http://hydra.usc.edu/gxe) with input of following variables: case-control study design, significance level (alpha) >0.05 (2 sided), model of inheritance was log additive, allele frequency was 0.28, and the genetic effect for odds ratio (OR) was 1.65 or greater. The present study achieved 80% of the statistical power. The chi square test was used to analyze any deviation from the Hardy–Weinberg equilibrium in controls. A binary logistic regression model was used to estimate the risk as the OR at the 95% confidence interval. Haplotypes of each individual consisting of 3 SNPs in *DR4* and 2 SNPs in *Casp5* were constructed, and the maximal likelihood haplotype frequencies were estimated using the expectation-maximization algorithm using the Arlequin program, version 2.000. The statistical analysis was done using the Statistical Package for Social Sciences software, version 11.5 (SPSS, Chicago, IL), and P 0.05 was considered statistically significant.

Results

Characteristics of subjects

A total of 225 controls and 200 cases were recruited for this study. There was no significant age difference between the cases (58.5 ± 12.4years) and the controls (56. 8 ± 10.8years) ($p = 0.117$). The cases had significantly higher percentage of smokers (69.9%) than the controls (22.7%) ($p < 0.001$). The demographic details of the study subjects and clinical characteristics of the patients are presented in Table 1.

DR4 and Casp3, 5 gene polymorphisms in bladder cancer

The genotype and allele frequencies of *DR4* and *Casp3, 5* gene polymorphism in healthy controls and patients with BC are presented in Table 2. The genotype frequency in the controls was in Hardy Weinberg Equilibrium. Individuals with *DR4 Arg141His* (AA) genotype were at higher risk of bladder cancer ($p = 0.036$; OR = 2.51; 95%CI = 1.06–5.94). This effect was evident in case of A allele carrier (GA + AA) too, with border line risk ($p = 0.053$; OR = 1.57; 95%CI = 0.99–2.47). Overall no statistically significant association was observed in *DR4 Thr209Arg C > G* and *DR4 Ala228Glu C > A* polymorphism (Table 2).

In *Casp5Leu13Phe G > C*, significant association was observed with GC genotype ($p = 0.025$; OR = 1.78; 95%CI = 1.07–2.94) and also in GC + CC ($p = 0.026$; OR = 1.68; 95%CI = 1.03–2.66). C allele carriers in *Casp5Ala90Thr T > C* showed low risk of BC ($p = 0.036$; OR = 0.83; 95%CI = 0.90–1.56). While in the case of *Casp3 G > A*, AG ($p = 0.003$; OR = 2.11; 95% CI = 1.30–3.42), GG ($p = 0.050$; OR = 2.18; 95% CI = 0.99–4.76) and G allele ($p = 0.001$; OR = 2.12; 95%CI = 1.34–3.36) and its carrier AG + GG ($p < 0.001$; OR = 1.85; 95%CI = 1.38–2.48) have shown significant association with BC risk (Table 2).

Association of DR4 and Casp3, 5 genotypes with tumor stage/grade

The patients with similar stage but with different grades respond to treatment differently. Hence we stratified the patients into three categories according to stage/grade [TaG1 (low risk NMIBC), $TaG_{2,3}$ + $T1G_{1-3}$ (High risk NMIBC) and T2+ (muscle invasive)]. TaG_1 was taken as a reference. No significant association was observed statistically in any of the six polymorphisms with the tumor stages (Data not shown).

Table 2
DR4 and *Casp5, 3* gene polymorphisms in bladder cancer polymorphisms and susceptibility to bladder cancer.

Genotype	Controls, *n* (%), ($n = 225$)	Cases, *n* (%), ($n = 200$)	*p* value	Age–gender-smoking adjusted OR (95%CI)
DR4 Thr209Arg C > G (rs4871857)				
GG	97(43.1)	86(43.0)	Ref	Ref
CG	113(50.2)	97(48.5)	0.591	0.88(0.55–1.41)
CC	15(6.7)	17(8.5)	0.719	0.83(0.31–2.24)
CG + CC	128(56.9)	114(57.0)	0.568	0.87(0.55–1.38)
G allele	307(68.2)	269(67.3)	Ref	
C allele	143(31.8)	131(32.8)	0.762	1.05(0.78–1.40)
DR4 Arg141His G > A (rs6557634)				
GG	113(50.2)	87(43.5)	Ref	Ref
GA	99(44.0)	91(45.5)	0.129	1.44(0.90–2.31)
AA	13(5.8)	22(11.0)	0.036	2.51(1.06–5.94)
GA + AA	112(49.8)	113(56.5)	0.053	1.57(0.99–2.47)
G allele	325(72.2)	265(66.3)	Ref	
A allele	125(27.8)	135(33.8)	0.060	1.33(0.99–1.77)
DR4 Ala228Glu C > A (rs20576)				
AA	91(40.4)	73(36.5)	Ref	Ref
AC	113(50.2)	105(52.5)	0.305	1.29(0.79–2.09)
CC	21(9.3)	22(11.0)	0.348	1.44(0.67–3.09)
AC + CC	134(59.6)	127(63.5)	0.247	1.32(0.83–2.09)
A allele	295(65.6)	251(62.8)	Ref	
C allele	155(34.4)	149(37.3)	0.394	1.13(0.85–1.50)
Casp5Leu13Phe G > C (rs3181320)				
GG	96(42.7)	75(37.5)	Ref	Ref
GC	93(41.3)	84(42.0)	0.025	1.78(1.07–2.94)
CC	36(16.0)	41(20.5)	0.199	1.51(0.81–2.82)
GC + CC	129(57.3)	125(62.5)	0.026	1.68(1.03–2.66)
G allele	285(63.3)	234(58.5)	Ref	
C allele	165(36.7)	166(41.5)	0.149	1.23(0.93–1.62)
Casp5Ala90Thr T > C (rs507879)				
TT	83(36.9)	65(32.5)	Ref	Ref
TC	97(43.1)	87(43.5)	0.380	1.26(0.76–2.08)
CC	45(20.0)	48(24.0)	0.217	1.48(0.79–2.77)
TC + CC	142(63.1)	135(67.5)	0.245	1.32(0.83–2.12)
T allele	263(58.4)	217(54.3)	Ref	
C allele	187(41.6)	183(45.8)	0.036	0.83(0.90–1.56)
Casp3G > A (rs4647603)				
AA	129(57.3)	72(36.0)	Ref	Ref
AG	80(35.6)	104(52.0)	0.003	2.11(1.30–3.42)
GG	16(7.1)	24(12.0)	0.050	2.18(0.99–4.76)
AG + GG	96(42.7)	128(64.0)	0.001	2.12(1.34–3.36)
A allele	338(75.1)	248(62.0)	Ref	
G allele	112(24.9)	152(38.0)	<0.001	1.85(1.38–2.48)

Association of DR4 and Casp3, 5 genotypes with smoking

We evaluated the gene smoking interaction to study the modulation of BC risk with respect to *DR4* and *Casp* gene polymorphisms. The patients were grouped as non-smokers and smokers. In *DR4 Ala228Glu C > A*, AC genotype was associated with higher risk of BC ($p = 0.035$; OR = 2.09). In the other five polymorphisms, no significant association was observed (data not shown). When controls non-smokers were compared with patients smokers, only *Casp5Leu13Phe G > C* showed significant association. GC showed three folds risk ($p < 0.001$; OR = 3.71) and CC showed two folds risk ($p = 0.019$; OR = 2.35) of BC (Table 3).

Association of DR4 and Casp haplotypes with bladder cancer risk

The ability of haplotypes to further substantiate the detection of association over the single locus analysis incited us to analyze haplotypes and their association with UBC susceptibility in *DR4* and *Casp.* Eight haplotype combinations were possible from haplotype analysis of *DR4*. In case of *DR4*, G-G-A was taken as reference. The haplotype results demonstrated that *DR4* (*Thr209Arg–Arg141His–Ala228Glu*) C-G-C to be associated with 1.8 folds (OR = 1.85; 95%CI = 1.05–2.81; $p = 0.033$) increased risk in bladder cancer patients (Table 4). There were a total of four possible haplotypes derived from *Casp* but overall no association was observed in any of four combinations.

Modulation of genotype variants and outcome after BCG immunotherapy

To analyze the association of *DR4* and *Casp* gene polymorphisms and risk of recurrence in NMIBC patients,

Table 3
Association of *DR4* and *Casp3, 5* with smoking status in controls non-smokers and patient smokers.

Genotype	Control Non-smokers *n* (%), ($n = 174$)	Patient Smokers, *n* (%),($n = 109$)	*p* value	Age–gender-smoking adjusted OR (95%CI)
DR4 Thr209Arg C > G (rs4871857)				
GG	70(40.2)	48(44.0)	Ref	Ref
CG	93(53.4)	52(47.7)	0.424	0.82(0.50–1.35)
CC	11(6.3)	9(8.3)	0.717	1.19(0.46–3.10)
DR4 Arg141His G > A (rs6557634)				
GG	95(54.6)	47(43.1)	Ref	Ref
GA	70(40.2)	53(48.6)	0.095	1.53(0.93–2.52)
AA	9(5.2)	9(8.3)	0.163	2.02(0.75–5.43)
DR4 Ala228Glu C > A (rs20576)				
AA	73(42.0)	41(37.6)	Ref	Ref
AC	81(46.6)	59(54.1)	0.317	1.29(0.78–2.16)
CC	20(11.5)	9(8.3)	0.620	0.80(0.33–1.92)
Casp5Leu13Phe G > C (rs3181320)				
GG	92(52.9)	28(25.7)	Ref	Ref
GC	54(31.0)	61(56.0)	<0.001	3.71(2.12–6.50)
CC	28(16.1)	20(18.3)	0.019	2.35(1.15–4.79)
Casp5Ala90Thr T > C (rs507879)				
TT	62(35.6)	44(40.4)	Ref	Ref
TC	82(47.1)	37(33.9)	0.105	0.64(0.37–1.10)
CC	30(17.2)	28 (25.7)	0.404	1.32(0.69–2.50)
Casp3G > A (rs4647603)				
AA	99(56.9)	54(49.5)	Ref	Ref
AG	59(33.9)	43(39.4)	0.269	1.34(0.80–2.23)
GG	16(9.2)	12(11.0)	0.446	1.38(0.61–3.12)

Table 4
Haplotype analysis of *DR4* and *Casp5* gene polymorphisms in bladder cancer patients and healthy controls.

	Controls *n*(%)	Patients *n*(%)	OR (95%CI)	*p* value
DR4 haplotype (Thr209Arg–Arg141His–Ala228Glu)				
G-G-A	146 (32.4)	125 (31.3)	Ref	Ref
G-G-C	72 (16.0)	54 (13.5)	0.88 (0.57–1.34)	0.543
G-A-A	41 (9.1)	44 (11.0)	1.25 (0.77–2.04)	0.364
G-A-C	49 (10.9)	46 (11.5)	1.09 (0.69–1.75)	0.700
C-G-A	83 (18.4)	48 (12.0)	0.68 (0.44–1.04)	0.073
C-G-C	24 (5.3)	38 (9.5)	1.85 (1.05–2.81)	0.033
C-A-A	25 (5.6)	34 (8.5)	1.59 (0.90–2.81)	0.111
C-A-C	10 (2.2)	11 (2.8)	1.29 (0.53–3.13)	0.581
Casp5 haplotype (Leu13Phe–Ala90Thr)				
G-T	165 (36.7)	127 (31.8)	Ref	Ref
G-C	120 (26.7)	107 (26.8)	1.16 (0.82–1.64)	0.408
C-T	98 (21.8)	90 (22.5)	1.19 (0.83–1.72)	0.347
C–C	67 (14.9)	76 (19.0)	1.47 (0.99–2.20)	0.059

further analysis was restricted to NMIBC patients only ($n = 148$). The median follow-up of NMIBC patients was 14 months (3–60 months). We analyzed the association of genotypes and risk of recurrence after BCG immunotherapy. We grouped patients into BCG treated ($n = 78$) and non-treated ($n = 70$) as these were patients of low grade tumors and did not require BCG immunotherapy.

The prospective effects of variant genotypes of *DR4* and *Casp* on the outcome after BCG treatment are illustrated in (Table 5). According to the age, gender, and smoking adjusted multivariate Cox regression hazards model, the individuals of BCG group with GG genotype of *Casp3 G > A* polymorphism showed increased risk of recurrence ($p = 0.009$; HR = 5.20; 95%CI = 1.51–17.96). No statistically significant association was observed with any other SNP.

Since increased recurrence risk for BC patients on BCG immunotherapy was observed, Kaplan–Meier recurrence free survival analysis was conducted. Subsequently, Kaplan–Meier analysis of *Casp3 G > A* showed recurrence free survival of 30 months for GG genotype as compared to 42 months for AA genotype carrying patients Fig. 1. Log rank *p* value was however, not significant (log rank $p = 0.239$).

Table 5
Influence of *DR4* and *Casp5, 3* gene polymorphisms on the risk of recurrence in BCG treated NMIBC patients.

	No Recurrence *n* (%)	BCG Recurrence *n* (%)	*p* value	HR[a] (95%CI)
DR4 Thr209Arg C > G				
GG	16(36.4)	16(47.1)	Ref	
CG	26(59.1)	14(41.2)	0.263	0.59 (0.23–1.50)
CC	2(4.5)	4(11.8)	0.852	1.17 (0.22–6.27)
CG + CC	28(63.6)	18(52.9)	0.334	0.65 (0.27–1.57)
DR4 Arg141His G > A				
GG	22(50.0)	16(47.1)	Ref	
GA	17(38.6)	17(50.0)	0.512	1.36 (0.54–3.45)
AA	5(11.4)	1(2.9)	0.827	0.79 (0.10–6.39)
GA + AA	22(50.0)	18(52.9)	0.604	1.26 (0.52–3.07)
DR4 Ala228Glu C > A				
AA	18(40.9)	14(41.2)	Ref	
AC	24(54.5)	15(44.1)	0.772	0.87 (0.33–2.28)
CC	2(4.5)	5(14.7)	0.311	2.04 (0.51–8.15)
AC + CC	26(59.1)	20(58.8)	0.871	1.08 (0.45–2.58)
Casp5Leu13Phe G > C				
GG	17(38.6)	15(44.1)	Ref	
GC	16(36.4)	16(47.1)	0.369	1.54 (0.60–3.97)
CC	11(25.0)	3(8.8)	0.451	0.55 (0.11–2.62)
GC + CC	27(61.4)	19(55.9)	0.726	1.17 (0.49–2.78)
Casp5Ala90Thr T > C				
TT	17(38.6)	8(23.5)	Ref	
TC	19(43.2)	20(58.8)	0.501	0.71 (0.27–1.90)
CC	8(18.2)	6(17.6)	0.241	0.42 (0.10–1.77)
TC + CC	27(61.4)	26(76.5)	0.381	0.65 (0.25–1.70)
Casp3G > A				
AA	20(45.5)	12(35.3)	Ref	
AG	22(50.0)	16(47.1)	0.387	1.56 (0.57–4.27)
GG	2(4.5)	6(17.6)	0.009	5.20 (1.51–17.96)
AG + GG	24(54.5)	22(64.7)	0.124	2.05 (0.82–5.14)

[a] HR, Age, gender and smoking adjusted Hazards Ratio; 95%CI, Confidence interval.

Discussion

Apoptosis and cancer

Apoptosis is the cell's intrinsic program to death, which plays an important role in physiologic growth control and homeostasis Malfunction of apoptosis plays an important role in the pathogenesis of tumors. Tumor cell survival could be induced by inactivation of proapoptotic signaling or activation of anti-apoptotic pathways. It is biologically plausible that the combined effects of single nucleotide polymorphisms in key apoptotic genes and exposure to environmental factors such as tobacco/cigarette smoking may influence an individual's risk of cancer.

Death receptors

Apoptosis can be triggered by death receptors (DRs), without any adverse effects. DRs are the members of tumor necrosis factor (TNF) receptor super family, known to be involved in apoptosis signaling, independent of p53 tumor suppressor gene. Selective triggering of DR-mediated apoptosis in cancer cells is a novel approach in cancer therapy. *DR4* mediates ligand-induced apoptosis; some data indicate that *DR4* may be involved in predisposition to tumor development.[11,17]

DR4 polymorphism in cancer

DR4 mutations have been described in different cancers, such as lung, head and neck cancer, non-Hodgkin's

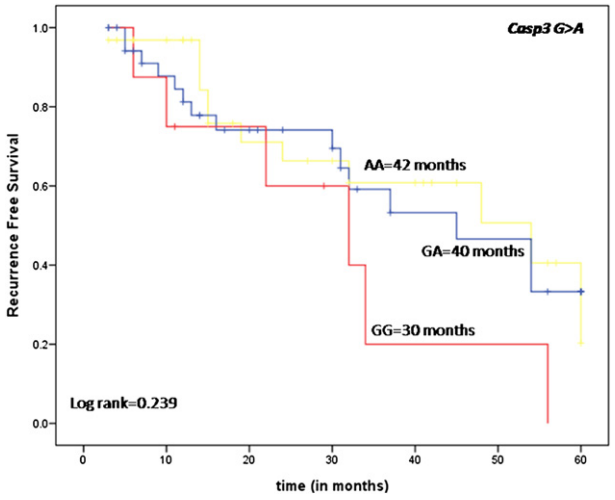

Figure 1. Kaplan–Meier survival analysis for BCG treated BC patients in relation to *Casp3* G > A polymorphism, Median recurrence free survival for AA = 42 months and GG = 30 months, log rank p value = 0.239.

lymphoma as well as in breast cancer, which suggest *DR4* to be an attractive candidate of tumor suppressor gene.[10–12] Several genotyping studies have addressed the association of *DR4* Thr209Arg in different types of cancer, but the results have been controversial[7,11,18]

Similarly, we did not find association of *DR4 Thr209Arg C > G* and *DR4 Ala228Glu C > A* polymorphism with the BC risk which was compatible to the observations made by Ulybina et al., 2009 in lung cancer[8] and in breast cancer.[17] Conversely, Fisher et al., 2001 reported significant risk of lung cancer in *DR4 Thr209Arg C > G.*[11] In another study by Frank et al., 2006, a significant association of the *DR4 Thr209Arg* variant was reported with a decreased colorectal cancer risk.[19]

These data provide the first large-scale molecular epidemiological evidence that the *DR4* polymorphism is associated with environmental exposure and bladder cancer risk, possibly through modulating the capacity of the receptor ligand complex to engage the apoptotic pathway.

In addition, another *DR4* polymorphism (A1322G, K441R) in the death domain of *DR4*, has been reported to be functionally linked to resistance against TRAIL-induced apoptosis in several cancer cells.[20]

Caspases polymorphism in cancer

We also evaluated some of the caspases which are assumed to be the executioner of apoptosis in human cancers. The biological function of the caspase-5 protein is poorly understood. Caspase-5 is believed to play a role in various aspects of inflammation[21]; in addition, Casp5 gene was repeatedly shown to be a target for mutation in a subset of human cancers.[22] In our study, significant risk was observed in GC genotype and also in GC + CC of *Casp5Leu13Phe G > C*. In *Casp5Ala90Thr T > C,* C allele carriers were at higher risk of BC which is also consistent with another study by Linda et al., 2009 in renal cancer in European population.[23] Contrarily Ulybina et al., 2009 did not find any significant association with *Casp5*.[8]

Further, our result demonstrated that SNP (G > A) found in 5′ UTR region of Caspase 3 was significantly associated with increased risk with BC. Similar results were observed by Chen et al., 2008 in squamous cell carcinoma.[16] Accumulating evidence suggest that genetic polymorphisms in the promoter region may affect transcription.[24] We believe that, that 5′ UTR is likely to contribute to transcription activity of Caspase 3 that may influence the gene expression, thereby contributing to risk of BC. Heterozygous genotype of *Casp3* also showed risk with smoking when compared between smokers and non-smokers patients. Heterozygous and variant genotype of *Casp5Leu13Phe G > C* also demonstrated risk of BC when compared between controls non-smokers and patient smokers.

Because individual polymorphisms are likely to confer modest effects to the risk of BC, we examined the effects of *DR4* and *Casp* gene polymorphisms by performing haplotype analyses. In *DR4* haplotypes, C-G-C was associated with 1.8 folds (OR = 1.85; 95%CI = 1.05–2.81; $p = 0.033$) increased risk in bladder cancer patients. Functional effect of these haplotypes in a large cohort in various ethnicities is warranted. Nevertheless, these results suggest that an appropriate combination of *DR4* and *Casp* polymorphisms modify the risk of overall and invasive BC.

Survival

We analyzed the association of *DR4* and *Casp* polymorphisms with risk of recurrence in NMIBC patients. The NMIBC patients were categorized on the basis of BCG treatment in BCG group and no BCG group. According to the age, gender, and smoking adjusted multivariate Cox regression hazards model, the individuals of BCG group with GG genotype of *Casp3 G > A* polymorphism showed increased risk of recurrence ($p = 0.009$; HR = 5.20; 95%CI = 1.51–17.96). No statistically significant association was observed with any other SNP.

Since increased recurrence risk for BC patients after BCG immunotherapy was observed, Kaplan–Meier recurrence free survival analysis was conducted. Subsequently, Kaplan–Meier analysis of *Casp3 G > A* showed recurrence free survival of 30 months for GG genotype as compared to 42 months for AA genotype carrying patients Fig. 1. Log rank p value was however, not significant (log rank $p = 0.239$).

Conclusion and limitations

While adjudging the inference of our observations limitations and sources of bias should also be considered. Case-control studies may be subjected to selection bias and recall bias. The biological role for many of the *DR4* and *Casp* in BC and the functional effects for many of the SNPs are still unknown. Therefore, these novel findings require further

research and replication in larger studies and well defined high throughput advanced techniques like Real time PCR or Microarray. In summary, our results suggested an association between *DR4* and *Casp* polymorphisms and BC risk. These results, upon validation and confirmatory advanced techniques, may one day be used as a risk assessment tool in understanding the etiopathlogy of BC.

Conflict of interest statement

None.

Acknowledgments

The study was funded by Department of Science and Technology, New Delhi, Govt. of India.

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