

# Death Receptor 4 and Bladder Cancer Risk<sup>1</sup>

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## Abstract

**Tumor necrosis factor-related apoptosis-inducing ligand stimulates the extrinsic apoptotic pathway by binding to death receptors 4 (DR4) and 5 (DR5). In DR4 exon 4, a C→G polymorphism at amino acid 626 located immediately 3' to one of the main receptor ligand interface regions, results in a threonine→arginine change. We found that the DR4 exon 4 G/G genotype was associated with an overall decreased risk of bladder cancer in Caucasians [odds ratio (OR) = 0.58; 95% confidence interval (CI), 0.38–0.88]. This protective effect was more apparent in younger individuals (OR = 0.42; 95% CI, 0.20–0.87) than in older individuals (OR = 0.60; 95% CI, 0.35–1.02) and in women (OR = 0.45; 95% CI, 0.20–0.99) than in men (OR = 0.60; 95% CI, 0.36–0.99). Moreover, the protective effect was greater for light smokers (OR = 0.19; 95% CI, 0.06–0.59) compared with heavy smokers (OR = 0.83; 95% CI, 0.41–1.69). 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.**

## Introduction

Resistance to apoptosis, imparting a survival advantage to cancer cells, is a recognized cause of tumorigenesis. Members of the TNF<sup>3</sup> family and their cognate ligands play critical roles in cell death. APO2L/TRAIL is a subfamily of TNF receptor gene superfamily with broad tissue expression and involvement in the extrinsic apoptotic pathway (1–7). APO2L/TRAIL binds with equal affinity to four different cell surface receptors, two proapoptotic receptors: DR4 (DR4 or APO2L/TRAIL R1), TRAIL-receptor 2 (APO2L/TRAIL-R2, DR5, KILLER/DR5); and two decoy receptors (not capable of inducing cell death): TRID and DcR2 (3–4). It has been suggested that the regulation of APO2L/TRAIL-induced death is restricted by receptor expression and binding (1–7).

DR4, located on chromosome 8p21, was the first DR for APO2L/TRAIL to be identified (3). The DR4 type I membrane protein contains 486 amino acids, composed of two extracellular cysteine-rich pseudorepeats. Two receptor loops, the 50- and 90-s loops, mediate most of the ligand receptor interactions. One loop consists of mostly a hydrophobic motif that is conserved in ligand receptor complexes throughout the TNF superfamily, and the other loop is unique for each individual complex and appears to be used to control receptor selectivity and cross-reactivity (8). The principle elements of the DR4 ligand-binding domain are encoded by exons 3 and 4 (8, 9).

Trimerization of the cysteine-rich extracellular domain of DR4 with the APO2L/TRAIL ligand recruits death domain-containing adaptor

proteins such as FADD and TRADD. The adaptor protein also contains a death effector domain that mediates a homotypic interaction with initiator procaspases 8 or 10, which activates effector caspase 3, cleaves poly(ADP-ribose) polymerase, and degrades the DNA. Caspase 3 is the executioner of the cell, responsible for the last phase of the cell's destiny, the deliberate disassembly of the cell into apoptotic bodies (10–12).

Recently, a C→G single nucleotide polymorphism was identified in exon 4 of the DR4 gene. Fisher *et al.* (9) found the 626 C→G polymorphism in the ectodomain of the DR4 gene by direct sequencing of genomic DNA from tumor and normal samples. Cycle sequencing of DR4 exon 4 in 31 lung cancer cell lines revealed that 21 (68%) of the cell lines had a C→G substitution at nucleotide 626. Although the functional effect of this polymorphism is presently unknown, its location immediately 3' to one of the main receptor ligand interface regions suggests that it may influence receptor ligand binding and initiation of the apoptotic pathway (9). This polymorphism results in the substitution of an arginine for threonine. In addition, DR4 has been shown to be up-regulated in response to DNA-damaging agents (13). The purpose of this investigation was to examine the association of the DR4 polymorphism and bladder cancer risk. We hypothesized that the DR4 variant allele genotypes modulate bladder cancer risk.

## Materials and Methods

**Case and Control Recruitment.** We genotyped 468 subjects (253 bladder cancer patients and 215 healthy control subjects) who had participated in an ongoing case-control study (14). The case subjects had histologically confirmed bladder cancer without previous radiotherapy or chemotherapy and were enrolled when initially seen at The University of Texas M. D. Anderson Cancer Center and The Methodist Hospital (Houston, TX) between 1999 and 2002. The control subjects had no history of cancer and were selected from a contact database of the Kelsey-Seybold Foundation, the largest managed-care organization in the Houston, Texas, metropolitan area. Details of the subject recruitment and study design are previously described by Zhao *et al.* (14). The institutional review boards of The University of Texas M. D. Anderson Cancer Center, the Kelsey-Seybold Foundation, and The Methodist Hospital approved this study.

**Genotyping Methods.** The RFLP-PCR method was used to amplify the polymorphic region of DR4 exon 4 (GenBank accession no. 18570975) and distinguish the variant alleles stated above. Genomic DNA used for this assay was extracted from peripheral blood lymphocytes. The PCR primers used were forward, 5'-ATCCTCTGGGAAGACTCTGTGG-3', and reverse, 5'-GGGGA-CAGGCAGATGGAC-3' (Genesis, The Woodlands, TX). Briefly, the RFLP-PCR reaction was performed in a 25- $\mu$ l reaction mixture containing 2.5  $\mu$ l of 10 $\times$  PCR buffer [500 mM KCl and 100 mM Tris-HCl (pH 9.0), 2.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2.0 mM (2.5  $\mu$ l) deoxynucleoside triphosphates, 4  $\mu$ l of each primer (1.25  $\mu$ M), 100 ng of template DNA, and 1.0  $\mu$ l of *Taq* polymerase (5 units/ $\mu$ l) in storage buffer B (Promega)]. The PCR cycling conditions for the assay were 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 5 min. After PCR amplification, a 15- $\mu$ l aliquot of the PCR product was digested overnight at 37°C in 18  $\mu$ l of reaction containing 1  $\mu$ l of *Dra*III enzyme. The digestion product was resolved on a 3.5% agarose gel stained with ethidium bromide at for 1 h. Three genotypes created by the polymorphic site were identified (Fig. 1): the CC homozygous (200 + 100-bp bands); CG the heterozygote (300 + 200 + 100-bp bands); and GG the homozygous (300-bp band).

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<sup>3</sup> The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; OR, odds ratio; CI, confidence interval; H-W, Hardy-Weinberg.

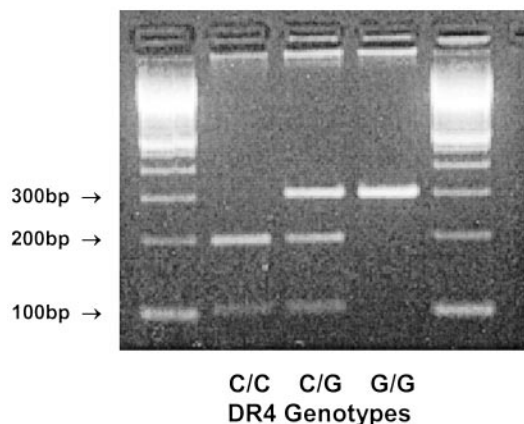


Fig. 1. Variant genotypes of the DR4 C626G polymorphism. RFLP-PCR based genotyping of the C626G polymorphism. Lane 1: C/C homozygous genotype; Lane 2: C/G heterozygous genotype; and Lane 3: G/G homozygous genotype.

**Statistical Analysis.** Intercooled STATA 7.0 was used to perform the statistical analysis. In the first step of the analysis, Pearson's  $\chi^2$  test was used to compare the distribution of select demographic variables such as sex, ethnicity, smoking status, and the DR4 genotypes in the case and control subjects. Student's *t* test was used to compare mean age and the mean pack-years smoked between cases and control subjects. The cases were stratified into three categories of smoking: never; former; and current smokers. An ever smoker was one who had smoked at least 100 cigarettes in his or her lifetime, and a former smoker was one who had stopped smoking at least 1 year before diagnosis for the cases and 1 year before the interview for the controls. A light smoker was one who had smoked < 20 years (the median number of years in the control population), whereas a heavy smoker was one who had smoked > 20 years. Pack-years smoked were calculated using the following formula: pack-years = (number of cigarettes/day/20 cigarettes)  $\times$  number of years smoked. H-W equilibrium was tested by using the goodness-of-fit  $\chi^2$  test to compare the observed allele frequencies with the expected frequencies determined from control subjects.

In the second step of the analysis, ORs and 95% CIs were used to estimate risk associated with the DR4 genotypes by using both univariate and unconditional multivariate logistic regression models; in the latter model, adjusting for confounding factors such as age, sex, and smoking status. *P*s < 0.05 were considered statistically significant.

## Results

Our study included 253 confirmed Caucasian bladder cancer case patients, of whom 74.3% were men and 25.7% were women. Among these cases, 29.7% were never smokers, 47.4% were former smokers, and 22.9% were current smokers. In our control population of 215 healthy individuals, we had 67.9% men and 32.1% women. Among these controls, 51.7% were never smokers, 40.9% were former smokers, and 7.4% were current smokers.

The distribution of C/C, C/G, and G/G genotypes differed significantly between cases and controls (*P* = 0.026). The protective G allele frequency was found less frequently in bladder cancer cases than in controls. Compared with the C/C genotype, the C/G and G/G genotypes were associated with reduced bladder cancer risk. In the cases, the frequencies of the homozygous variant (C/C), the heterozygous variant (C/G), and the homozygous variant (G/G) were 15.4, 60.1, and 24.5%, respectively; in the controls, the frequencies were 15.7, 49.0, and 35.3%, respectively. There was a departure from the H-W equilibrium among cases (*P* = 0.001), whereas controls exhibited H-W equilibrium (*P* = 0.820).

To compare the effect of the threonine (CC and CG) amino acid with the arginine (GG) amino acid, the CC and CG genotypes were combined into one category and compared it with the GG genotype

(Table 1). After adjustment by age, sex, and smoking status, the GG genotype was associated with an overall significant protective effect (OR = 0.55; 95% CI, 0.36–0.84) compared with individuals with CG and CC genotypes. This protective effect was more apparent in younger individuals (OR = 0.42; 95% CI, 0.20–0.87) than in older individuals (OR = 0.60; 95% CI, 0.35–1.02), and in women (OR = 0.45; 95% CI, 0.20–0.99) than in men (OR = 0.60; 95% CI, 0.36–0.99). Moreover, the protective effect was greater for light smokers (OR = 0.19; 95% CI, 0.06–0.59) compared with heavy smokers (OR = 0.83; 95% CI, 0.41–1.69). We also observed attenuation of the protective effect with an increasing history of cigarette pack-years. In addition, there was a joint effect between the DR4 genotypes and smoking status in bladder cancer risk (Fig. 2). The referent group for this analysis was never smokers with the G/G genotype. Among individuals with the CC and CG genotypes, we found ORs of 1.57 (95% CI, 0.82–3.03), 3.2 (95% CI, 1.63–6.30), and 12.68 (95% CI, 4.75–33.88) for never smokers, former smokers, and current smokers, respectively. We observed a reduction in risk for the individuals with the GG genotype. The ORs were 1 for never smokers, 1.68 (95% CI, 0.77–3.70) for former smokers, and 2.21 (95% CI, 1.25–3.93) for current smokers with the GG genotype.

## Discussion

It is biologically plausible that the combined effects of single nucleotide polymorphisms in key apoptosis genes and exposure to environmental factors such as tobacco may influence an individual's

Table 1 Risk estimates for the variant DR4 genotypes among Caucasians<sup>a</sup>

Genotypes	Cases (N)	Control (N)	Univariate OR (95% CI)	Adjusted OR** (95% CI)
Overall	253	215		
CG + CC	191	139	1	1
GG	62	76	0.59 (0.40–0.89)	0.55 (0.36–0.84)
Gender				
Male				
CG + CC	140	96	1	1
GG	48	50	0.66 (0.41–1.06)	0.60 (0.36–0.99)
Female	65	69		
CG + CC	51	43	1	1
GG	14	26	0.45 (0.21–0.96)	0.45 (0.20–0.99)
Age				
<62				
CG + CC	70	62	1	1
GG	21	33	0.56 (0.30–1.07)	0.42 (0.20–0.87)
$\geq$ 62				
CG + CC	121	77	1	1
GG	41	43	0.61 (0.36–1.07)	0.60 (0.35–1.02)
Smoking status				
Never	75	111		
CG + CC	65	73	1	1
GG	19	38	0.65 (0.34–1.25)	0.64 (0.33–1.23)
Light				
CG + CC	35	29	1	1
GG	5	21	0.20 (0.07–0.59)	0.19 (0.06–0.59)
Heavy <sup>b</sup>				
CG + CC	100	37	1	1
GG	38	17	0.83 (0.42–1.64)	0.83 (0.41–1.69)
Pack-year by quartile <sup>c</sup>				
<5.6				
CG + CC	15	13	1	1
GG	2	11	0.16 (0.03–0.85)	0.15 (0.02–0.93)
5.6–20.0				
CG + CC	20	16	1	1
GG	3	10	0.24 (0.06–1.02)	0.22 (0.41–1.06)
20.0–40.5				
CG + CC	43	20	1	1
GG	15	7	1.00 (0.35–2.83)	0.91 (0.30–2.73)
$\geq$ 40.5				
CG + CC	57	15	1	1
GG	23	2	0.69 (0.27–1.72)	0.73 (0.28–1.90)

<sup>a</sup> Adjusted by age, gender, and smoking status (where appropriate).

<sup>b</sup> Heavy smokers = smoked >20 years.

<sup>c</sup> For ever smokers only.

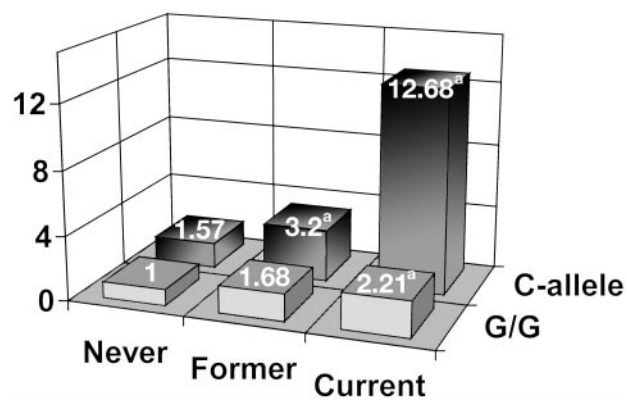


Fig. 2. Joint effects of DR4 genotypes and smoking status in Caucasians. Adjusted by age, gender, and smoking status. <sup>a</sup> =  $P < 0.05$ .

risk of cancer. The C→G single nucleotide polymorphism in exon 4 of the *DR4* gene was identified by Fisher *et al.* (9). In this first large-scale molecular epidemiological study of a polymorphism in the extrinsic apoptotic pathway and bladder cancer, we found that the *DR4* exon 4 G/G genotype was associated with an overall decreased risk of bladder cancer in Caucasians. We also found that this genotype had a larger protective effect in younger patients than in older patients. A probable explanation is that our older patients have a greater risk accumulated from environmental exposures, which would overwhelm even a resistant genotype. Tobacco smoke contains procarcinogenic compounds that are metabolized into reactive intermediates, resulting in DNA damage. An example of this is the conversion of benzo(*a*)pyrene to benzo(*a*)pyrene-diol epoxide, a highly reactive intermediate. It has been shown that DNA damage increases DR4 transcription (15), which elicits the apoptotic cascade (3). Therefore, *DR4* may exert its protection upon exposure to carcinogens. Our data demonstrated greatest protective effects among light smokers than in heavy smokers. This corroborates data reported for the *p53* gene (16) in relation to cancer susceptibility, supporting the idea that heavy carcinogenic exposure may overwhelm genetic effects. Genetic differences in risk are less evident at elevated doses of carcinogens when dire environmental effects such as heavy tobacco exposure may override underlying genetic predisposition.

In addition, stratification by gender revealed a more pronounced significant protective effect of the G/G genotype for women. It has been suggested that at the same level of exposure, women are more susceptible to DNA damage (adduct formation) than men are (17, 18). However in our study, we found the opposite result. This may be because the women in our study population did not have the same level of exposure as the men in this study population because the women were more likely to be light smokers and younger individuals.

In summary, we present data suggesting that the *DR4* polymor-

phism in exon 4 is associated with the risk of bladder cancer, possibly by modulating *DR4*'s apoptotic potential. Because DR4 has been shown to be up-regulated in response to DNA-damaging agents, its association with environmental exposures may be particularly important. We plan to confirm these data in larger studies and determine the mechanism by which variant genotypes modify cancer risk.

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