

Association of Caspase-8 Genotypes With Bladder Cancer Risk

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Abstract. Background/Aim: Rs3824129 is a functional six-nucleotide insertion(I)/deletion(D) polymorphism in the promoter region of caspase 8, an essential apoptosis gene. We aimed to examine the association of this polymorphism with the risk of bladder cancer in the Taiwanese population. Materials and Methods: Caspase-8 rs3834129 genotypes were determined and their associations with bladder cancer risk were evaluated among 375 patients and 375 controls by the PCR-RFLP methodology. In addition, the interaction of caspase-8 rs3834129 genotypes with personal behaviors and clinicopathological features were examined. Results: The frequencies of II, ID and DD genotypes for caspase-8 rs3834129 were non-differentially distributed between the two groups (p for trend=0.7187). Analysis of allelic frequency distribution also indicated that the D variant allele was not associated with a risk of bladder cancer. There was no obvious joint interaction between caspase-8 rs3834129 genotypes and smoking, alcohol consumption, and clinical

stage and grade. Conclusion: Caspase-8 rs3834129 genotypes play a minor role in the personal susceptibility to bladder cancer in Taiwan.

Bladder cancer is the sixth and seventeenth most commonly diagnosed cancer among men and women in the world, respectively, with nearly 500,000 newly diagnosed cases in 2018 (1). In Taiwan, the incidence of bladder cancer has been steadily increasing and currently occupy the ninth and fourteenth places among the common types of cancer for men and women, respectively (2). The high incidence has been suggested to be closely associated with a combinative effect of smoking, exposure to chemicals and PM2.5, prior radiation therapy, and frequent bladder infections, in addition to family history and genetic factors (3, 4). Interestingly, the contribution of genetic factors and the underlying etiology of bladder cancer remains largely unrevealed. In the past several years, although useful biomarkers for early detection of bladder cancer have been proposed (5-12), the practical genomic markers in clinics are still urgently needed and the mechanisms and signaling pathways warrant further investigations.

Apoptosis, also named as programmed cell death, is a process that removes unwanted cells from the body, with multiple cellular alterations including shrinkage of the cell, blebbing of the membrane, condensation of chromosome, and fragmentation of nucleus (13, 14). In the literature, mounting evidence has reported that the loss of homeostasis in the apoptosis program is associated with cancer initiation, progression, and treatment resistance (14, 15).

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A family of proteins known as caspases are the engines of apoptosis. There is mounting evidence linking the genetic variations in caspase genes with carcinogenesis. For instance, disruption of *Apaf-1*, mutation in *caspase-10*, and frameshift mutations in *caspase-5* are closely associated with Noonan's Syndrome, autoimmune lymphoproliferative syndrome type II and hereditary gastrointestinal and endometrial tumors, respectively (16-18). The contribution of genetic variations of *caspase-8*, whose protein plays an important role in the extrinsic apoptosis pathway, to cancer susceptibility has also been examined in a few cancers (19-29), including neuroblastoma (22), lung cancer (23), breast cancer (24-26), prostate cancer (27), colorectal cancer (28, 29), and oral cancer (30, 31).

One of the most commonly studied polymorphisms in *caspase-8* is rs3834129 (-652, 6N ins/del), a six-nucleotide insertion/deletion polymorphism, which has been functionally characterized to lead to the under-expression of caspase-8 mRNA (21). As for bladder cancer, it was reported that *caspase-8* rs3834129 ins/del and del/del genotypes were statistically associated with lower bladder cancer risk in a Chinese population (32), but not in an Indian population (33). In 2017, a meta-analysis showed that *caspase-8* rs3834129 polymorphism was associated with decreased risks for esophageal, lung, breast, gastric, colorectal, cervical, renal, and most of all, bladder cancers, and overall cancer in Asian populations (34). No study has evaluated this polymorphism in relation to bladder cancer susceptibility in Taiwan. Thus, in this study, we aimed at evaluating its association with bladder cancer risk, and examining the joint effects of *caspase-8* rs3834129 genotype with smoking behaviors in a representative Taiwanese population.

Materials and Methods

Population sampling and recruiting. This multi-year study has been approved by the Institutional Review Board of the China Medical University Hospital (DMR104-IRB-158) and each participant has completed a written-informed consent. All the clinical investigations and records in this study were restrictively performed according to the principles expressed in the Declaration of Helsinki. To sum up, 375 patients diagnosed with bladder cancer were recruited at the China Medical University Hospital. After communication between patients and doctors, all the recruited cases voluntarily participated, completed a comprehensive questionnaire and provided 3 to 5 ml of peripheral blood. The clinical characteristics of patients including histological details were abstracted from medical charts. Then, as a control, an equal number of non-cancer healthy subjects were selected by matching for age and gender after initial random sampling from the Health Examination Cohort visiting the hospital. They were asked in the same procedure as the cases. The exclusion criteria of the control group were defined as we published previously (5-10). Briefly, those with previous malignancy, metastasized cancer from another site or a tumor of unknown origin, and any familial or genetic diseases were excluded from the control group. As mentioned above, all participants completed a short

questionnaire regarding personal characteristics, especially their individual habits. Smokers were defined as daily or almost daily smokers who had smoked at least five packs of cigarettes per year in their lifetime. Age of smoking initiation, whether they were currently smoking or had already quit, and if so, when they had quit, and on average, how many cigarettes they smoked or had smoked daily were recorded for smokers. Overall, the selective demographic characteristics of all the investigated subjects are summarized in Table I.

Genotyping procedures for *caspase-8* rs3834129. The genomic DNA for each subject was extracted from his/her peripheral blood leukocytes using QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, ROC) accordingly, long-term stored at -80°C , 100-fold diluted and aliquoted in three Eppendorfs for *caspase-8* rs3834129 genotyping as a working stock at -20°C , as described in our previous publications (35, 36). The *caspase-8* rs3834129 genotyping methodology as for the designing of the primer pairs and the selection of restriction enzyme are concisely described below. First, the polymerase chain reaction (PCR) program was set as: 1) one hot-start cycle at 94°C for 5 min; 2) 35 repeated cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec, and 3) a final extension at 72°C for 10 min. Second, the oligo-nucleotide sequences of the forward and reverse primers for *caspase-8* rs3834129 genotyping were 5'-ACTCTGCATGCCAGGAGCTA-3' and 5'-CTGGGGAAG CCTCACTGTAT-3', respectively. Third, the PCR products were subject to full digestion with *Pvu II* restriction enzyme (New England Biolabs, Beverly, MA, USA). Finally, the digested DNA products were subject to 3% agarose gel electrophoresis for 25 min, staining in ethidium bromide and immediately observed under UVC irradiation. All the PCR-RFLP processing was repeated independently and blindly by at least two qualified researchers (Chang, Wang, Tsai, Tung or Li), and the genotyping analyzing results were 100% concordant among them.

Statistical methodology. The Student's *t*-test was used in age (a continuous variables) comparison between the two groups. The Pearson's chi-square was used in comparison for the distribution of the *caspase-8* rs3834129 genotypes among the subgroups according to the smoking status, etc. The associations between *caspase-8* rs3834129 genotypes and bladder cancer risk were estimated with individual odds ratios (ORs) plus 95% confidence intervals (CIs). Any comparison results with a *p*-value less than 0.05 was identified as statistically significant.

Results

Comparison of characteristics between the bladder cancer patients and the healthy controls. The distributions of age, gender, smoking and drinking status in addition to clinical bladder cancer stages and grades for the 375 bladder cancer patients and 375 non-cancer controls are summarized in Table I. As mentioned in Materials and Methods, we have adopted the frequency matching strategy in our choosing of the healthy controls. Thus, there was no obvious difference in the aspects of age or gender between the case and control groups (Table I). As for personal behaviors, cigarette smokers and alcohol drinkers were at non-significant higher levels in the case group than in that of control (both $p>0.05$)

Table I. Basic characteristics of the 375 patients with bladder cancer and 375 controls investigated in this study.

Characteristic	Controls (n=375)			Cases (n=375)			p-Value
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			62.9 (9.8)			61.4 (10.3)	0.7315 ^a
Age group (years)							0.7108 ^b
≤55	152	40.5%		158	42.1%		
>55	223	59.5%		217	57.9%		
Gender							0.5525 ^b
Male	287	76.5%		279	74.4%		
Female	88	23.5%		96	25.6%		
Personal habits							
Cigarette smoking	186	49.6%		201	53.6%		0.3063 ^b
Alcohol drinking	176	46.9%		189	50.4%		0.3807 ^b
Stage							
Non-muscle-invasive				235	62.7%		
Muscle-invasive				140	37.3%		
Grade							
Low				151	40.3%		
High				224	59.7%		

SD: Standard deviation; ^abased on Student's *t*-test; ^bbased on Chi-square test.

Table II. Distributions of caspase-8 rs3834129 genotypic frequencies among bladder cancer patients and healthy controls.

Genotype	Cases, n (%)	Controls, n (%)	Adjusted OR (95%CI) ^a	p-Value ^b
II	216 (57.6)	205 (54.7)	1.00 (Reference)	
ID	137 (36.5)	146 (38.9)	0.89 (0.71-1.13)	0.4511
DD	22 (5.9)	24 (6.4)	0.88 (0.51-1.36)	0.6539
ID+DD	159 (42.4)	170 (45.3)	0.85 (0.71-1.12)	0.4183
<i>P</i> _{trend}				0.7187
<i>P</i> _{HWE}				0.7690

I: Insertion; D: deletion; OR: odds ratio; CI: confidence interval; HWE: Hardy-Weinberg Equilibrium; ^aData adjusted for confounding factors: age, gender, cigarette smoking and alcohol drinking status; ^bBased on chi-square test without Yates' correction.

(Table I, upper part). With regard to clinical features, 62.7% of the patients had non-muscle-invasive disease while 59.7% of the patients had high-grade tumors (Table I).

Association between caspase-8 promoter genotypes and bladder cancer risk. The comparisons about the distributions of caspase-8 rs3834129 II, ID and DD genotypes among the cases and controls are presented in Table II. Overall, the genotypes of caspase-8 rs3834129 were non-differentially distributed between the case and control groups (*p* for trend=0.7187) (Table II). In detail, heterozygous and homozygous variant ID and DD genotypes were not associated with altered bladder cancer risk compared with the wild-type II genotype (adjusted OR=0.89 and 0.88, 95%CI=0.71-1.13 and 0.51-1.36, *p*=0.4511 and 0.6539, respectively). In addition, in the dominant model, there was

no association between caspase-8 rs3834129 ID+DD genotypes and bladder cancer risk, compared with the wild-type II genotype (adjusted OR=0.85, 95%CI=0.71-1.12, *p*=0.4183). The distribution of caspase-8 rs3834129 genotypes in the control group fits well with the Hardy-Weinberg Equilibrium (*p*_{HWE}=0.7690).

We have also performed the analysis of allelic frequency distributions for caspase-8 rs3834129 using the methodology of chi-square test without Yates' correction, and the results are summarized in Table III. The results showed that caspase-8 rs3834129 genotypes were not associated with the risk of bladder cancer. In detail, the relative percentage of variant allele D was 24.1% (n=181 out of 750) in the case group, slightly but non-significantly lower than that of 25.9% (n=194 out of 750) in the control group (adjusted OR=0.89, 95%CI=0.74-1.12, *p*=0.4382) (Table III).

Table III. Allelic frequencies for caspase-8 rs3834129 polymorphisms among the bladder cancer patients and healthy controls.

Allele	Cases, n (%) (n=750)	Controls, n (%) (n=750)	Adjusted OR (95%CI) ^a	p-Value ^b
I	569 (75.9)	556 (74.1)	1.00 (Reference)	
D	181 (24.1)	194 (25.9)	0.89 (0.74-1.12)	0.4382

I: Insertion; D: deletion; OR: odds ratio; CI: confidence interval. ^aData adjusted for confounding factors: age, gender, cigarette smoking and alcohol drinking status. ^bBased on chi-square test without Yates' correction.

Table IV. Odds ratios for association of caspase-8 rs3834129 genotype with bladder cancer after stratification by smoking status.

Genotype	Non-smokers, n		OR (95%CI) ^a	aOR (95%CI) ^b	p-Value ^c	Smokers, n		OR (95%CI) ^a	aOR (95%CI) ^b	p-Value ^c
	Controls	Cases				Controls	Cases			
II	107	102	1.00 (ref)	1.00 (ref)		98	114	1.00 (ref)	1.00 (ref)	
ID	70	63	0.94 (0.61-1.46)	0.91 (0.68-1.33)	0.7057	76	74	0.84 (0.55-1.27)	0.89 (0.62-1.38)	0.4049
DD	12	9	0.79 (0.32-1.95)	0.85 (0.58-1.64)	0.6032	12	13	0.93 (0.41-2.14)	0.88 (0.59-1.33)	0.8664
Total	189	174				186	201			
ptrend					0.8619					0.7069

^aMultivariate logistic regression analysis; ^bmultivariate logistic regression analysis after adjusting for age, gender and alcohol drinking status; ^cChi-square without Yates' correction; insertion; D: deletion; CI: confidence interval; aOR: adjusted odds ratio.

Joint effects of caspase-8 rs3834129 genotypes and personal behavioral or clinical indexes on bladder cancer risk. We are interested in evaluating the joint effects of caspase-8 rs3834129 genotypes with personal behaviors such as cigarette smoking and alcohol drinking, and the results are shown in Tables IV. Firstly, among non-smokers, those carrying ID and DD genotypes at caspase-8 rs3834129 had 0.94- and 0.79-fold increased odds for bladder cancer risk (95%CI=0.61-1.46 and 0.32-1.95, $p=0.7057$ and 0.6032), respectively (Table IV, left panel). After adjusting for age, gender and alcohol drinking, the results remained non-significant for ID and DD genotypes (adjusted OR=0.91 and 0.85, 95%CI=0.68-1.33 and 0.58-1.64, respectively) (Table IV, left panel). Meanwhile, a non-significant effect was also found among smokers (Table IV, right panel). Secondly, among the subgroups of non-alcohol drinkers and alcohol drinkers, those carrying ID and DD genotypes at caspase-8 rs3834129 had a non-significant differential risk of having bladder cancer (data not shown). Lastly, among the subgroups of different stages and grades, those carrying ID and DD genotypes at caspase-8 rs3834129 did not have a significant differential risk of having bladder cancer either (data not shown).

Discussion

The cysteine-aspartic acid protease 8 is an evolutionarily conserved cysteine endoprotease, named for its capacity to

cleave peptide bonds after aspartic acid residues. It is responsible for initiating programmed cell death *via* activating caspase-3 in the apoptosis signaling cascade (37-39). Although down-regulation or loss of caspase-8 have been frequently reported in various types of tumors, such as neuroblastoma (40, 41), medulloblastoma (42) and relapsing glioblastoma (43) and small cell lung carcinoma (44), the role of inherited genetic variations of caspase-8 in cancer risk, progression and treatment responses are still debatable. Rs3834129 is the most commonly examined caspase-8 variant. In 2007, Sun and his colleagues reported that the del allele at caspase-8 rs3834129 was associated with a decreased susceptibility to multiple types of cancer, including esophageal, lung, breast, cervical, colorectal and gastric cancer (45). Subsequently, the genotypes of caspase-8 rs3834129 were reported to be associated with the risk for brain tumor (22), breast cancer (27), melanoma (46) and kidney cancer (47). Biologically, the del genotype has been reported to destroy a stimulatory protein 1 binding element in the promoter regulatory region, leading to decreased caspase-8 transcriptional activity and eventually suppression of T lymphocyte apoptosis (45). There were also some studies reporting no association between the genotypes of caspase-8 rs3834129 with various types of cancer, for instance, the oral and colorectal cancers in the Taiwanese population (31, 48).

This is the first study evaluating caspase-8 rs3834129 polymorphism in bladder cancer susceptibility in the Taiwanese population, however no significant association

was found. Our results are consistent with a previous one performed in a South Indian population reporting that there was no significant association between *caspase-8* rs3834129 genotype and bladder cancer risk among a population containing 212 bladder cancer patients and 250 controls (33). However, it was reported that *caspase-8* rs3834129 del/del and del/ins genotypes were associated with a lower risk of bladder cancer risk in a Chinese population (32). The sample sizes of ours and Wang's study (32) are much similar, and both of the distributions of *caspase-8* rs3834129 fit the HWE well in the control groups. The distribution of *caspase-8* rs3834129 genotypes in the two control groups was not statistically different. The discrepant observation may come from different sampling methodologies or environment exposure, but not from differences in the genotyping methodology used or in the genetic background.

We also analyzed the possible joint interaction between *caspase-8* rs3834129 genotypes and several risk behaviors such as cigarette smoking and alcohol drinking. There was no interaction between *caspase-8* rs3834129 genotypes and smoking or alcohol drinking on determining the personal risk to bladder cancer (Table IV). In 2009, Wang and his colleagues reported that there was a significant interaction between *caspase-8* rs3834129 genotypes and smoking on bladder cancer risk (32). The interaction of *caspase-8* rs3834129 genotypes and smoking on bladder cancer risk warrants further investigations. We have also examined the correlations between genotypes of *caspase-8* rs3834129 and clinicopathological features of the 375 bladder cancer patients. The results showed that there was no influence of *caspase-8* rs3834129 on bladder cancer risk among patients of early or late stages, with either a low or higher grade.

To sum up, this study provided evidence that *caspase-8* rs3834129 was not associated with bladder cancer risk in this population of 375 cases and 375 controls. In addition, we found no altered risk among people with or without cigarette smoking or alcohol drinking. Moreover, *caspase-8* rs3834129 variant genotypes were not associated with clinical stage or grade. The contribution of *caspase-8* genotypes to bladder cancer needs to be studied in additional, ethnically diverse populations.

Conflicts of Interest

All the Authors declare no conflict of interest regarding this study.

Authors' Contributions

Research Design: Chen M, Tsai CW and Tsai YT; Patient and Questionnaire Summarize: Shih LC and Shen TC; Experiment Performance: Wang YC and Chang WS; Statistical Analysis: Tsai YT and Lin ML and Chao CY; Manuscript Writing: Chen M, Tsai CW and Bau DT; Reviewing and Revising: Bau DT, Chang WS and Tsai CW.

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