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Functional Genetic Variants in Apoptosis-associated *FAS* and *FASL* Genes and Risk of Bladder Cancer in a Turkish Population

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Abstract. *Background: The present study aimed to evaluate the role of functional polymorphisms of apoptosis-associated Fatty acid synthase (FAS) and fatty acid synthase ligand (FASL) genes in bladder cancer susceptibility as first presentation in a Turkish population. Patients and Methods: Genotypes of 91 patients with bladder cancer and 101 healthy controls were evaluated for the polymorphism of FAS-1377 G/A and FASL-844 T/C genes by polymerase chain reaction and restriction fragment length polymorphism analysis. Results: The frequency of the FAS-1377 G allele was significantly higher in patients with bladder cancer compared to controls (p<0.001). A significantly increased risk for developing bladder cancer was found for the group bearing a T allele for FASL-844 compared to the homozygous FASL-844 CC genotype (p=0.027). FAS-1377 GG genotype and FASL-844 T allele were found to be independently associated with an increased risk of bladder cancer. Additionally, gene–gene interaction analysis revealed that the frequency of FAS-1377AA with FASL-844TC was significantly lower in patients with bladder cancer in comparison to those of controls (p<0.001). Extensive studies for gene–gene interaction are still needed. Conclusion: Our study provides new evidence that FAS-1377 G and FASL-844 T alleles may be used as low-penetrant risk factors for bladder cancer development in a Turkish population.*

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Bladder cancer is the fourth most common type of cancer among men and eighth among women worldwide, with the highest incidence rates in Western countries and the lowest in Asian countries (1). In Turkey, bladder cancer is the third most common type of cancer among men, being the second most common cancer of the genitourinary tract after prostate cancer with high morbidity and mortality (2). The etiology of bladder cancer appears to be multi-factorial, with exogenous environmental factors and endogenous molecular factors playing possible roles. Identification and validation of novel prognostic molecular biomarkers of bladder cancer, as well as causative oncogenic therapeutic markers, are ofcritical importance for the early management and cure of bladder cancer (3).

Apoptosis is the physiological mechanism of programmed cell death that plays an important role in diverse biological processes, such as normal tissue development and maintenance of tissue homeostasis, and elimination of cancer cells (4, 5).

FAS; also known as Tumor Necrosis Factor Receptor Superfamily member 6 (TNFRSF)/cluster of differentiation 95 (CD95)/apoptosis antigen 1 (APO-1) is a cell surface receptor and a potent member of the death receptor family which is expressed in a variety of tissues. The native ligand to FAS, FASL (also known as TNFSF6/CD95LG) is a type-II transmembrane protein that belongs to theTNF superfamily. FASL interacts with its surface receptor FAS to activate the death signal cascade, which results apoptotic cell death (6-9).

Alterations of FAS/FASL expression may promote malignant transformation and progression by reducing the apoptotic function of cells (10). Several studies have shown that functional polymorphisms in low-penetrant genes can alter gene expression or enzymatic activities and can, thus,

affect the risk of cancer initiation, developmentand progression. The human *FAS* genes (GenBank accession no:AY450925) and *FASL* (GenBank accession no:Z96050) are mapped tochromosomes 10q24.1 and 1q23, respectively. Functional polymorphisms have been identified in the promoter region of the *FAS* and *FASL* genes: *FAS*-1377G/A (rs2234767) and *FASL*-844T/C (rs763110). Functional study has revealed that the basal expression of *FASL* gene in individuals carrying the *FASL*-844C allele is significantly higher than that in those carrying the *FASL*-844T allele (11).

In the past decade, many molecular epidemiological studies in different populations have demonstrated the associations between *FAS* and *FASL* functional polymorphisms and risk of cancer, including of the bladder, brain, breast, cervix, lung, pancreas, stomachand malignant melanoma, esophageal squamous cell carcinoma, and acute myeloid leukemia (18-22). Meta-analyses have reported that single-nucleotide polymorphisms (SNPs) of *FAS*/*FASL* apoptosis-related genes might be associated with increased risk of some types of cancer (12-16).

Hence, the aim of this study was to investigate the effect of the promoter polymorphisms of *FAS*-1377G/A and *FASL*-844T/C and their association with the risk of developing bladder cancer in a case–control study in a Turkish population. We hypothesized that these genes involved in the cell death pathway might have a role as low penetrance genesin susceptibility to bladder cancer.

Materials and Methods

Study population. The hospital-based prospective case–control study included 91 patients with bladder cancer. Eligible cases were patients newly-diagnosed with histopathologically confirmed transitional cell carcinoma of the bladder between January 2006 and December 2010 in our high-volume tertiary Center. Tumors were staged according to the 2002 TNM classification and the 2004 WHO grading system (24, 25). Patients who had previous radiotherapy and chemotherapy were not included in the study. A total of 101 healthy individuals were selected for the healthy control group. All controls were age- and sex-matched and had no evident malignancy or chronic disease. This routine data collection approved by the Institutional Ethics Committee (approval number: VGH4-1/2009), and blood samples were collected only within formed consent.

Genotyping. A 10-ml of sample of venous blood was collected from each participant into a test tube containing EDTA. Genomic DNA was extracted from peripheral whole-blood according to a salting-out technique. *FAS* and *FASL* polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. The primers used for *FAS*-1377 gene region were: Forward: 5'-TGTGTGCACAAGGCTGGCGC-3' and reverse: 5'- TGCATCTGTCACTGCACTGCACTTACCACCA-3'; those for *FASL*-844gene region were: forward: 5'-CAGCTACTCGGAGGCCAA-3' and reverse: 5'-GCTCTGAGGG GAGAGACCAT-3'. For amplification, 25 μl PCR mixture was separately prepared for each gene containing approximately 100 ng of template DNA, 0.5 μl of each primer, all four deoxyribonucleoside 5' triphosphates (each at 0.2 mM), 2.5 mM $MgCl_2$ and 1 U Taq polymerase in 1× reaction buffer (Fermentas UAB Inc, Vilnius, Lithuania) PCR conditions were: an initial melting step of 45 s at 95˚C; followed by 35 cycles of 45 s at 94˚C, 45 s at 65˚C and 45 s at 72˚C; and a final elongation step of 5 min at 72˚C. The restriction endonuclease BstUI (Bsh1236I) was used to determine the *FAS*-1377 gene polymorphism. The products of this reaction were separated on agarose gels containing ethidium bromide. BstUI digestion generated 104- and 18-bp fragments for *FAS*-1377 G allele and 122-bp fragments for *FAS*-1377 A allele. The restriction endonuclease BseMI (BsrDI) was used to determine the *FASL*-844 gene polymorphism. The products of this reaction were separated on agarose gels containing ethidium bromide. *Bse*MI digestion generated an uncut 401-bp fragment for *FASL*-844 T allele and two 233-bp and 168-bp fragments for *FASL*-844 C allele.

Statistical analysis. The SPSS software (version21, SPSS Inc, Chicago, IL, USA) was used for data analysis. Clinical parameters are stated as the mean±SD. Mean values were compared among participants by the unpaired Student's *t*-test. Differences in the distribution of genotypes and alleles between patients and controls were tested using chi-square (χ^2) test and Fischer's exact test. The odds ratios (OR) and the confidence interval (CI) were calculated to estimate the relative risk. Multivariate logistic regression analysis was performed with binary logistic regression. Risk factors that appeared to be potential significant predictors in single-variate analysis were included in the multiple logistic regression models. This analysis was used to identify association of FAS and FASL polymorphisms among several independent factors. In the logistic regression model, patient and control groups were used as the dependent variable. The model included gender, smoking habit, *FAS* and *FASL* polymorphisms as independent variables. Values of $p<0.05$ were considered statistically significant.

Results

Characteristics of the study groups. A total of 91 patients with bladder cancer and 101 controls were recruited for the study. The demographic details of the study participants and clinical characteristics of the patients are described in Table I. The mean age of the patients with bladder cancer and healthy controls were 63.9±13.3 and 61.9±6.2 years, respectively. No significant differences were found between patients and controls in terms of median age ($p=0.194$). There were significant differences with regard to gender, with there being more men in the study than in the control group ($p<0.001$). In addition, the patient group had a significantly higher percentage of smokers than the control group ($p<0.001$).

Association between FAS and FASL polymorphisms and risk of bladder cancer. The allelic and genotypic frequencies for *FAS*-1377 and *FASL*-844 polymorphisms in patients with bladder cancer and controls are given Table II. To find potential associations, we tested each single variant, as well as each pair of variants, against cases and controls.

Table I. *Demographic details of bladder cancer patients and healthy controls.*

Variables	Patients n (%)	Controls n (%)	*p*-Value
Gender			
Female	19 (20.9)	54 (53.5)	<0.001
Male	72 (79.1)	47 (46.5)	
Age (years),mean±SD	63.9±13.3	61.9±6.2	0.194
Smoking status			
Non-smokers	19 (20.9)	84 (83.2)	<0.001
Smokers	72 (79.1)	17 (16.8)	
Tumor stage			
Superficial (pTa-pT1)	71 (78)	-	
Invasive (pT2-pT4)	20 (22)	-	
Grade			
Low grade	49 (53.8)	-	
High grade	42 (46.2)	-	
Event			
Non-recurrence	36 (39.6)	-	
Recurrence	55 (60.4)	-	

Values are reported as number (n) of patients (percentage of the total group). *p*-Values less than 0.05 denote statistical significance.

Table II. *Genotypic and allelic frequencies forthe Fatty Acid Synthase (FAS) and Fatty Acid Synthase Ligand (FASL) polymorphisms among patients with bladder cancer and controls.*

SNP	Controls n (%)	Patients n (%)	*p*-Value
FAS -1377 G/A rs (2234767)			
GG	52 (51.5)	67 (73.6)	<0.001
GA	20 (19.8)	21 (23.1)	
AA	29 (28.7)	3 (3.3)	0.003
*GA+AA	49 (47.5)	24 (26.4)	
G allele	124 (61.3)	155 (85.1)	<0.001
A allele	78 (38.7)	27 (14.9)	0.003
FASL -844 T/C rs (763110)			
TT	18 (17.8)	17 (18.7)	0.078
TC	50 (49.5)	57 (62.6)	
CC	33 (32.7)	17 (18.7)	0.027
**TT+TC	68 (67.3)	74 (81.3)	
T allele	86 (42.6)	91 (50)	0.027
C allele	116 (57.4)	91 (50)	0.878

Values are reported as number (n) of patients (percentage of the total group). *p*-Values less than 0.05 denote statistical significance. SNP: Single nucleotide polymorphism; OR: odds ratio; CI: confidence interval. Comparison of *GA+AA genotype *versus* GG genotype, OR=0.555, 95% CI 0.372-0.828; **TT+TC genotype *versus* CC genotype, OR=1.208, 95% CI 1.021-1.428.

Firstly, we evaluated the *FAS*-1377 polymorphism group. The frequency of the G allele was found to be significantly higher in patients compared to controls (χ^2=22.265; OR=1.357, 95% CI 1.192-1.544, p<0.001). Because the variant AA genotype was rare in this study population, we combined the variant AA genotype and with the GA genotype, assuming a dominant genetic model. A significant increased risk for developing bladder cancer was found for those with GG genotype compared with the A carriers (χ^2=9.137; OR=1.403, 95% CI=1.123-1.753), (p=0.003) (Table I).

The observed genotypic frequencies of *FASL*-844 in both groups were in agreement with Hardy–Weinberg equilibrium (χ^2=5.100, p=0.078). When *FASL*-844 polymorphism allelic frequencies were compared in the study groups, carriers of the T allele also had an increased risk of bladder cancer (χ^2=4.866; OR=1.208, 95% CI 1.021-1.428) (p=0.027) (Table II).

In multivariate analysis, potential risk factors in the single-variate analysis, including male gender, smoking, *FAS*-1377 GG genotype and *FASL*-844T allele-bearing genotype were includedin the multiple logistic regression models. The genotypic distributions of *FAS*-1377 and *FAS*-844 SNPs were consistent with the Hosmer and Lemeshow test. Multivariate logistic regression analysis was performed to correct the ORs for the genotype to examine whether the *FAS*-1377 GG genotype and *FASL*-844 T carriers were independently associated with an increased risk of bladder cancer after adjusting for the effects of gender and smoking (OR=2.216, 95% CI 1.028-4.779 p=0.042 for FAS-1377 GG genotype; OR=2.222, 95% CI 1.091-4.522, p=0.028 for FASL-844 TT+TC genotype).

Gene–gene interaction between FAS-1377 G/A and FASL-844 T/C polymorphisms. We also analyzed gene–gene interactions by different combinations to evaluate the synergistic effect on bladder cancer. The results are shown in Table III. The combination of *FAS*-1377 GG and *FASL*-844 TC was the most frequent haplotype, observed at 44% and 27.7% in patients and healthy controls, respectively. Combined genotype analysis showed that the frequency of the haplotype with the combination of *FAS*-1377 AA and *FASL*-844 TC was significantly lower in the patient than in the control group (χ^2=11.002, OR=0.192, 95% CI 0.060-0.612, p=0.001). However, the other genotypes did not contribute to risk of bladder cancer in comparison to the combination of *FAS*-1377 GG and the *FASL*-844 TC (Table III).

Association between FAS and FASL polymorphisms and clinicopathological characteristics of bladder cancer. Distributions of *FAS*-1377and *FASL*-844 genotypes according to clinical parameters and tumor characteristics of bladder cancer patients were examined.

Out of the 91 patients with bladder cancer, 49 (53.8%) had a low-grade tumor, the remaining 42 (46.2%) had a high-

Table III. *Combined genotypic frequencies forthe FAS and FASL polymorphisms among the patients with bladder cancer and controls.*

Combined genotype		Controls n (%)	Patients n (%)	χ^2	*p*-Value	OR (95% CI)*
FAS -1377 G/A	FASL -844 T/C					
GG	TC	28 (27.7)	40 (44)			1.000
	TT	8 (7.9)	13 (14.3)	0.063	0.801	1.104 (0.510-2.389)
	CC	16 (15.8)	13 (14.3)	1.607	0.205	0.675 (0.365-1.245)
GA	TT	5 (5)	5 (5.5)	0.278	0.598	0.733 (0.231-2.328)
	TC	8 (7.9)	13 (14.3)	0.063	0.801	1.104 (0.510-2.389)
	CC	7 (6.9)	4 (4.4)	1.256	0.262	0.515 (0.158-1.682)
AA	TT	4 (4)	0 (0)	5.294	0.035	-
	TC	16 (15.8)	3 (3.3)	11.002	0.001	0.192 (0.060-0.612)
	CC	9 (8.9)	0 (0)	11.017	0.001	-

*Compared to FAS-1377 GG and FAS-844 TC combination. OR: Odds ratio; CI, confidence interval. *p*-Values less than 0.05 denote statistical significance.

grade tumor. Carriers of the FAS-1377 A allele genotypes more frequently had low-grade tumors compared to those with GG genotype in the patient group, but the difference was not statistically significant (p=0.142). At the same time, there were no significant differences in histological tumor grade by *FASL*-844 genotype in the patient group (p=0.319).

A total of 71 patients (78%) had a superficial tumor (pTa-pT1), and the remaining 20 (22%) had an invasive tumor (pT2-pT4). *FAS*-1377 and *FASL*-844 genotype exhibited no apparent relationship with tumor type of bladder cancer (p=0.084 and p=0.412, respectively).

Discussion

At the time of initial diagnosis, about 70% to 75% of bladder tumors are non-muscle invasive bladder cancer and the remaining 25% to 30% are muscle-invasive bladder cancers. Non-muscle-invasive bladder cancer can be treated successfully with transurethral resection and intravesical therapies, but on progression to muscle-invasive disease, radical cystectomy may be considered as an initial treatment option. Bladder-preserving approaches, concomitant treatment with a complete transurethral resection and radiotherapy, alone or with concomitant platinum-based combination chemotherapy, are reasonable alternatives to cystectomy for patients who are medically unfit for surgery (22, 23).

Immunotherapy is one of the most promising treatment strategies for cancer. Gene therapy may soon help improve these therapies by inhibiting tumor growth *via* immunomodulatory mechanisms. However, bladder cancer is known to correlate with abnormal metabolic pathways and molecular instability.

The TNF family are monocyte-derived cytokines that have been implicated in tumor regression, septic shock, and cachexia. FAS–FASL is a cytokine and is involved in cell death (26). However, the function of *FAS*/*FASL* genes in urothelial cancer has not been clearly explained. Possible associations with the polymorphism of genes of the apoptosis pathway have rarely been investigated in Turkey. Herein, gene–gene interaction ofthe *FAS* and *FASL* genes was also analyzed, as well as their polymorphism. FAS receptor is known to be widely expressed in the tissues, but FASL is especially expressed on cells of the immune system, such as activated T-cells and natural killer cells. However, it is now known that FASL is present in many other cell types in various organs, such as the brain, testicles, placenta and eyes. Reduced expression of FAS or increased expression of FASL are known to exist in some cancer types. Lack of cell surface FAS expression is one of the main routes of apoptotic resistance in tumor formation and progression (6).

In the present prospective case–control study, we investigated the *FAS*-1377G/A polymorphism, which impairs apoptotic signal transduction and has been demonstrated to be associated with elevated risk of developing various types of human cancers (12, 27, 28). Meta-analyses investigated the relationship between this polymorphism and cancer risk in recent years. Zhong-Xing and co-workers applied a meta-analysis of 17,858 cases and 24,311 controls for *FAS*-1377G/A SNP from 44 case–control studies. This study indicated that *FAS*-1377 G allele was protective against cancer (18). Similar associations were detected by different research groups. Qui *et al*. produced a meta-analysis of 10,564 cancer cases and 12,075 controls including *FAS*-1377G/A SNP from 17 case–control studies (19). Zhang *et al*. investigated 11,461 cancer cases and 12,708 controls concerning this polymorphism from 34 published case–control studies (20). The researchers found that the *FAS*-1377 G allele was associated with a statistically reduced

risk of cancer in Asians but not Caucasians and Africans in the subgroup analysis by ethnicity (18-20). Conversely, Hsu *et al*. reported that the *FAS*-1377A allele was a protective factor against developing cancer in a Chinese population (14). Similarly, our present data shows that individuals carrying the *FAS*-1377 G allele were at an increased risk for developing bladder cancer and that the *FAS*-1377 A allele had a protective effect. This finding is consistent with results for *FAS*-1377G/A polymorphism in primary brain tumor (13). These inconsistent results among the different ethnicities may indicate different effects of the *FAS*-1377 G/A polymorphism on bladder cancer risk in different ethnic backgrounds. Furthermore, we suggest that *FAS*-1377G/A polymorphism may play different roles among different tumor types.

Some population studies also indicated an association between the *FASL*-844T/C functional polymorphism and increased risk of several types of cancer (12, 26, 27). Lately, Xu *et al*. performed a meta-analysis of 19,810 cancer cases and 23,485 controls including *FASL* rs763110 SNP from 47 case–control studies (17). This study showed that the FASL-844 C allele was associated with cancer development, especially in the Asian population. Zhang Z *et al*. determined an approximately 1.5-fold higher risk of bladder cancer for patients who have *FASL*-844 CC genotype when compared to those with *FASL*-844 TT or TC (21). On the other hand, Ter-Minassion *et al*. revealed that those under the age of 60 years with the *FASL*-844 TT or TC had higher risk for non-small cell lung cancer compared to those with *FASL*-844 CC genotype in stratification analysis according to age (29). Similarly, a significant increased risk for developing bladder cancer was found in *FASL*-844T carriers compared with those with the *FASL*-844 CC genotype in our case–control study,although some studies on cancer risk denoted no association between *FASL*-844T/C SNP and various types of cancer such as melanoma, cervical and thyroid cancer (30-32). Compared to published data, meta-analyses show that the genotype distributions of the *FASL*-844T/C polymorphism vary with ethnicity (29, 30). For the *FASL*-844T/C polymorphism, the frequencies of the T allele were 28.4%, 36.9% and 62.8% in Asians, Caucasians and Africans, respectively (17). Our data showthat differences in genetic and environmental background can appear among different ethnicities. Hence, additional studies are required to verify possible ethnic differences in the effect of *FAS* and *FASL* functional SNPs on cancer pathogenesis.

Single-gene studies are likely to provide limited value in evaluating bladder cancer risk. Therefore, we investigated the association between bladder cancer risk and gene–gene interaction between the *FAS* and *FASL* genes. Among all the possible combinations analyzed, *FAS*-1377 AA and FASL-844 TC gene combination was associated with protective association against bladder cancer.

Conclusion

To the best of our knowledge, the data acquired in this study confirmed for the first time the association of the *FAS* and *FASL* gene variants in the promoter regions with bladder cancer in a Turkish population. These findings suggest that *FAS*-1377 G and *FASL*-844 T alleles may be used as low-penetrant risk factors for bladder cancer development in the Turkish population. Gene–gene interaction analysis revealed that the *FASL*-1377 AA genotype may have predominant effect on the decreased risk of bladder cancer over the *FAS*-844 T/C SNP. Rigorous larger-sample size studies with population-based and more detailed clinical information about tumors are required to validate this association more comprehensively. Future functional studies are important to evaluate genotype–phenotype correlation and the influence of *FAS* and *FASL* gene polymorphisms on the expression levels in bladder cancer tissue samples of patients.

Conflicts of Interest

None.

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