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Association of *B7-H4* gene polymorphisms in urothelial bladder cancer

Asuman ÖZGÖZ[1], Murat ŞAMLI[2], Deniz DİNÇEL[3], Ahmet ŞAHİN[2], Ümit İNCE[4], Yeşim SAĞLICAN[4], Faruk BALCI[3], Hale ŞAMLI[3,*]

[1]Department of Nutrition and Dietetics, Fazıl Boyner Faculty of Health Sciences, Kastamonu University, Kastamonu, Turkey
[2]Department of Urology, Faculty of Medicine, Acıbadem University, İstanbul, Turkey
[3]Department of Genetics, Faculty of Veterinary Medicine, Uludağ University, Bursa, Turkey
[4]Department of Pathology, Faculty of Medicine, Acıbadem University, İstanbul, Turkey

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Background/aim: We aimed to study polymorphisms of the *B7-H4* gene in order to evaluate a possible association in urothelial carcinoma, as it is highly expressed in cancer tissues.

Materials and methods: In this study *B7-H4* gene rs10754339, rs10801935, and rs3738414 SNPs were studied by PCR-RFLP method in paraffin-embedded tumor specimens from 62 urothelial carcinoma patients and in a control group including 30 patients without bladder cancer.

Results: We detected that the rs10754339 polymorphism was more frequent in the cancer patients when compared with the control group ($P < 0.05$). Only the rs3738414 polymorphism showed a statistically significant difference in frequency between pathologic diagnostic groups.

Conclusion: The rs10754339 AA genotype distribution was found to have a higher frequency whereas the rs3738414 AG genotype distribution was lower in the bladder cancer group ($P < 0.05$). None of the genotype distributions showed a significant difference from the control group for the rs10801935 polymorphism. We conclude that *B7-H4* has the potential to be a useful prognostic marker in urothelial carcinoma.

Key words: Bladder cancer, polymorphism, *B7-H4* gene

1. Introduction

Bladder cancer is a very severe malignancy threatening life quality. Every year 300,000 new bladder cancer cases are reported (1). Urothelial bladder cancer has different properties at clinical, pathological, genetic, and epigenetic levels (2). B7-like molecules play a critical role in the control and regulation of antigen-specific immune response by binding to their receptors in T cells (3). B7 family members B7-H1, B7-DC, B7-H2, B7-H3, and B7-H4 act together in order to perform overlapping functions of regulating the priming, proliferation, and maturation of T cells (4,5) and forming immune responses, one of them being tumors, to their many targets (6,7). Interestingly, B7 molecules can both stimulate and suppress T-cell activation (8,9). The negative signals that suppress T-cell activation are generally provided by B7 members B7-H1 and B7-H4 (10). It has been found that B7-H4, in many tissues and cells, controls the host's inherent immune response by neutrophil progenitor growth suppression, and furthermore it has an effect on the response of T cells. The human *B7-H4* gene is located on 1p13.1; it has six exons and five introns covering 66 kb. The sixth exon can be alternatively spliced to form two different transcripts (11). *B7-H4* is able to functionally inhibit attachment of T cells, growth, cytokine secretion, and the development of cytotoxicity by inhibiting cell cycle progression (3). *B7-H4* expression was first observed in many cancer cells such as colon, prostate, lung, and fibrosarcoma (12,13) and human ovarian and lung cancer tissues (14). In studies performed, B7-H4 mRNA and protein was detected in all of the investigated 23 melanoma (15), 5 gastric cancer (16), and 6 nonsmall-cell lung cancer cell lines (17). Although *B7-H4* expression has been detected in various kinds of human cancer tissues (18), its role in urothelial tumors still remains unknown. The untranslated regions (UTRs) and introns, and the first intron most importantly, are able to regulate gene expression resulting in stable mRNA production, translational efficiency enhancement,

* Correspondence: halesamli@gmail.com

and degradation of mRNA. In Zhang et al.'s study, three newly detected single nucleotide polymorphisms (SNPs) in *B7-H4* gene UTRs and the first intron showed an association with breast cancer risk in the Han Chinese population (19). This was the first polymorphism study to report an association between *B7-H4* polymorphism and the risk of cancer (19). The second *B7-H4* polymorphism study in cancer, performed by Özgöz et al., was also in breast cancer (20), and to our knowledge there is no other polymorphism study, neither in breast cancer nor in any other types of cancers. Therefore, our study is the first *B7-H4* polymorphism study reporting an association between *B7-H4* polymorphism and the risk of bladder cancer.

2. Materials and methods

2.1. Patient and control groups

The study was conducted at Uludağ University, School of Veterinary Medicine, Department of Genetics and at Acıbadem University, School of Medicine, Department of Urology and Pathology in 2012 and 2013.

Ethical approval for the protocol was received from the Uludağ University Medical School's Research Ethics Committee (decision dated 31 May 2011, numbered 2011 – 12 / 6), and according to the Helsinki Declaration written informed consent was obtained from all the patients and the controls before performing the study.

Bladder cancer patients were selected randomly among the bladder cancer patients' list and classified according to their pathology. According to the WHO 2004 bladder cancer classification system, three groups of bladder cancer pathologies were selected for the study for their invasiveness and high progression ability; 19 low-grade papillary urothelial carcinomas (no muscle invasion was present), 20 high-grade papillary urothelial carcinomas (no muscle invasion was present), and 23 high-grade papillary urothelial carcinomas (muscle invasion was detected) were included. Thirty normal subjects were included in the study as controls.

2.2. DNA extraction and genotyping

In this study *B7-H4* gene rs10754339, rs10801935, and rs3738414 SNPs were studied by PCR-RFLP method in paraffin-embedded tumor specimens from 62 urothelial bladder cancer patients and in the blood of the control group consisting of 30 people.

For every patient 10 × 0.5 µm slides were prepared from paraffin blocks of bladder tumor specimens. Genomic DNA isolation was performed using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA, USA). In the control group, peripheral blood was collected in 2 mL EDTA tubes from the subjects and genomic DNA isolation was performed using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA).

In order to determine the rs10754339, rs10801935, and rs3738414 polymorphisms of the *B7-H4* gene, the genomic DNA was amplified using the following primers:

(F: 5′-TCCTATGGGTCTGTCAATG-3′,
R: 5′-GCTGCTAAACTCAAAGGC-3′),
(F: 5′-TAGTGGCGGTACAATAGC-3′,
R: 5′-AGTGCCTCTGTTTCTTCC-3′),
(F: 5′-AAAGACCTCACTGCTGTTCC-3′,
R: 5′-CCACAGTCAGGAGGAAAGTC-3′).

For rs10754339, the PCR conditions were as follows: 5 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C with a final extension at 72 °C for 10 min.

For the rs10801935 and rs3738414 polymorphisms, the PCR conditions were as follows: 5 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C with a final extension at 72 °C for 10 min.

The PCR products were detected on 2% agarose gel and then were digested with restriction enzymes MScI, SaII, and BtsI, and the digested products were detected on 3% NuSieve agarose gel.

2.3. Statistical analysis

Statistical analysis was conducted with SPSS 17.0 for Windows (Chicago, IL, USA). Data were compared by t-test and evaluated by Fisher's chi-square test.

3. Results

The patient and control groups were in a similar age interval (patient group 63.34 ± 12.5 years vs. control group 60.8 ± 11.1 years; $P > 0.05$). Male sex was more frequent among cancer patients (55 males vs. 7 females) and the same sex distribution was detected in the controls (28 males vs. 2 females) ($P > 0.05$). The patients were evaluated for the main risk factors of bladder cancer: smoking habit and chemical exposure. Forty-three of 62 urothelial bladder cancer patients were detected to be smokers ($P < 0.05$) and three of them were painters who were assumed to be exposed to some chemical solvents. It is also verified in our study that smoking habit is one of the most important risk factors causing bladder cancer.

We detected that the rs10754339 polymorphism was more frequent and the rs3738414 AG genotype was lower in cancer patients when compared with the control group ($P < 0.05$) (Table); only the latter SNP (rs3738414) showed a statistically significant difference in frequency between the 3 different pathologic diagnostic groups ($P < 0.05$). None of the genotype distributions showed a statistically significant difference from the control group for the rs10801935 polymorphism (Table).

Table. Genotype distribution of *B7-H4* gene polymorphisms in bladder cancer patient and control groups.

Groups	B7-H4 rs10754339 AA genotype frequency (n)	B7-H4 rs10754339 AG genotype frequency (n)	B7-H4 rs10754339 GG genotype frequency (n)
Patients (n = 62)	55	7	0
Controls (n = 30)	18	11	1
Groups	B7-H4 rs10801935 AA genotype frequency (n)	B7-H4 rs10801935 AC genotype frequency (n)	B7-H4 rs10801935 CC genotype frequency (n)
Patients (n = 62)	17	30	15
Controls (n = 30)	11	14	5
Groups	B7-H4 rs3738414 AA genotype frequency (n)	B7-H4 rs3738414 AG genotype frequency (n)	B7-H4 rs3738414 GG genotype frequency (n)
Patients (n = 62)	17	27	18
Controls (n = 30)	0	29	1

4. Discussion

In expression studies of *B7-H4*, it was seen that *B7-H4* is expressed in many cancers (21). In addition, such as the lung, testis, pancreas, prostate, placenta, uterus, skin, muscle, intestine, stomach, kidney, liver, heart, brain, and ovary, B7-H4 mRNA is also commonly expressed in many human peripheral tissues (3,12,22). It could be hypothesized that *B7-H4* expression is related to antitumor immunity downregulation, notably T-cell response (15). *B7-H4* expression in tumors is probably due to the abnormal posttranscriptional regulation (23). High Expression of *B7-H4* is associated with a decrease in cell apoptosis and enhancement in outgrowth of tumors. Variable N-glycosylation of *B7-H4* is reported and this probably leads to formation of a "barrier" mechanism in order to escape immunosurveillance (24). *B7-H4* is suggested to have a probable duty in transforming precancerous cells and making them escape from the immune system (23).

Because this gene is highly expressed in various cancer types, many *B7-H4* gene expression studies are available, but there are only two studies regarding gene polymorphism. The first polymorphism study performed by Zhang et al. was in breast cancer (19). According to the study of Zhang et al., *B7-H4* gene rs10754339, rs10801935, and rs3738414 SNPs could cause a risk of breast cancer and poor prognosis. In their study the rs10754339 AG genotype and G allele showed a high association that made them think that the G allele of rs10754339 may increase breast cancer risk. Zhang et al.'s study showed that some *B7-H4* alleles and genotypes may cause risks for developing and promoting breast cancer. In particular, the rs10754339 AG genotype and G allele (OR = 1.455, OR = 1.325) were mostly found in women with breast cancer and this SNP was thought to affect the forming of altered transcript kinds and gene expression (19).

The study of Özgöz et al. showed that, although statistically not significant ($P > 0.05$), the AG genotype of rs10754339 and the G allele frequency were higher in the breast cancer case group than the control group, and their study showed no association with the GG genotype (20). These results were compatible with the study of Zhang et al. (19). In contrast with these two studies, in our bladder cancer cases the rs10754339 AA genotype frequency was higher than in the control group ($P < 0.05$). Thus, it may be thought that the rs10754339 AA genotype is associated with bladder cancer rather than breast cancer. The rs10801935 CC genotype frequency in the breast cancer case group was lower than in the control group in the studies of both Zhang et al. and Özgöz et al. (19,20). Zhang et al. thought that the rs10801935 CC genotype may probably be protective in breast cancer patients (19). In contrast to these results, our study presented that bladder cancer cases had both higher CC and AA genotype rates, so this SNP may not be concluded to be useful in evaluating bladder cancer risk.

Again in the study of Zhang et al. (19), the *B7-H4* rs3738414 AA genotype and A allele were supposed to have a protective role in breast cancer, but in the study of Özgöz et al., this result was not verified, and in contrast, the GG genotype frequency was lower (20). In our study the rs3738414 AG genotype frequency was lower in the cancer case group than in the control group ($P < 0.05$), so the rs3738414 AG genotype may be protective in bladder cancer. Only the rs3738414 polymorphism showed a statistically significant difference in frequency between pathologic diagnostic groups ($P < 0.05$) in our bladder cancer case group.

To our knowledge our study is the first one reporting an association between *B7-H4* gene polymorphism and bladder cancer. *B7-H4* may be a potent marker for early cancer diagnosis but this needs to be verified by studies including various cancer types and larger case numbers from different study groups.

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