

CTLA-4 Exon One +49A/G Gene Variants in Patients with Superficial and Invasive Bladder Cancer: A Study in Southern Iran

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Abstract

Introduction: Cytotoxic T-cell lymphocyte antigen 4 (CTLA-4) is a member of the superfamily of immunoglobulins that are mainly expressed by activated T cells. It is established that blockade of CTLA-4 receptors leads to the enhancement of an immune response. Different polymorphisms of the CTLA-4 gene have been described which cause increased susceptibility to various malignancies such as lymphoma or multiple myeloma. Considering that bladder cancer is one of the most prevalent cancers worldwide, we have evaluated the role of CTLA-4 gene polymorphism at position +49 A/G in the formation or progression of bladder cancer in southern Iran.

Materials and Methods: A total of 226 individuals between February 2005 and June 2006 were included and placed into two subgroups: patients diagnosed with bladder cancer and a control group. Demographic data and risk factors were collected from both groups. The DNA of all subjects was extracted from their blood samples. Different genotypes of the CTLA-4 gene were determined using the restriction fragment length polymorphism (RFLP) technique and data were compared in both groups by using Pearson's chi-square test.

Results: The prevalence of AA, AG and GG genotypes at position 49, according to the PCR-RFLP method, were 57.5%, 37.2% and 5.3% in the control group, respectively. In the patient group, the prevalence of these genotypes was: AA in 57.5%, AG in 32.7% and GG in 9.8%. Statistical analysis of data showed no significant difference in both groups (P value=0.40). Also there was no correlation between different genotypes of the CTLA-4 gene and invasiveness of the disease in our cases.

Conclusion: Although polymorphism of the CTLA-4 gene at position 49 of exon-1 increases susceptibility to several malignancies, our study showed no relationship between polymorphism at this position and genetic susceptibility to the development of bladder cancer, nor was there any association with disease progression.

Keywords: Cytotoxic T-cell lymphocyte antigen 4, Polymorphism, Bladder cancer, Iran, Metastasis

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Introduction

Cytotoxic T-cell lymphocyte antigen-4 (CTLA-4), also known as CD152, is a member of the superfamily of immunoglobulins expressed mainly by activated T cells.¹ It is structurally similar to CD28. However, opposite to CD28, the CTLA-4-ligand interaction has an inhibitory effect on T-cell activation.² CTLA-4 plays an important role in the maintenance of peripheral tolerance as one of the key negative regulators of an adaptive immune response. Additionally it is established that blockade of CTLA-4 receptors leads to enhancement of the immune response.³⁻⁴ It may inhibit T-cell responses by antagonizing CD28-mediated signals.

T cells are known for their important antitumor immunity role and therefore molecules such as CTLA-4 that mediate regulation of T-cell activity could affect cancer susceptibility.⁵ Maker et al. showed that antibodies against CTLA-4 mediate cancer regression and autoimmunity in patients suffering from renal cancer and metastatic melanoma due to increased T-cell activation.⁶

More than 100 single-nucleotide polymorphisms have been identified in the CTLA-4 gene region, the most important of which are: -1722T>C, -1661A>G, -658C>T and -318C>T.⁷

The role of these polymorphisms on different types of cancers has been evaluated and it is established that different polymorphisms of CTLA-4 are associated with susceptibility to lymphoma, multiple myeloma, breast cancer, oral squamous cell carcinoma, human papilloma virus 16 related cervical cancers and renal cell carcinoma (RCC).⁸

Bladder cancer is the second most common genitourinary malignant disease in the USA and its risk increases with age, peaking between 50 and 70 years. Almost 69000 new cases of bladder cancer are diagnosed annually in the USA with 14000 deaths. In the Middle East, data reveal that more than 2000 people have been diagnosed with bladder cancer in Jordan and Egypt between 1996 and 2001 with a median age of 61.4.⁹

Although the role of CTLA-4 polymorphism is established in renal cell carcinoma, to our

knowledge there is no data available in the literature regarding CTLA-4 polymorphism and bladder cancer. In this case-control study we aim to evaluate the possible role of CTLA-4 gene polymorphism at position 49 of exon-1 and its susceptibility to bladder cancer or its progression to different stages of cancer in patients from southern Iran.

Materials and Methods

Patient and control groups

This study was carried out in Shiraz University of Medical Sciences hospitals from February 2005 to June 2006. There were 226 individuals registered who were divided into two groups: patient and control. The patient group consisted of 113 individuals who were selected from those who presented with symptoms of bladder cancer, such as gross hematuria. Each case underwent a careful physical examination, complete urine analysis, culture and urine cytology which was followed by ultrasonography and a CT scan of the urinary system. Their diagnosis of bladder cancer was later confirmed by cystoscopy. A transurethral resection of the bladder (TURB) or radical cystectomy was performed for patients based on clinical stage and treatment protocols. Bladder tissues were sent for histopathological study after surgery and a diagnosis of bladder cancer at different stages and types was confirmed.

There were 113 normal, healthy individuals selected as the control group. These individuals were matched for age and sex with the patient group. Demographic data of both groups were gathered. All individuals were consented prior to participating in the study. Each of the subjects from both groups had 10 cc of blood drawn and stored at -20°C in an EDTA tube for DNA extraction.

Genotyping

DNA was extracted according to the modified method of Olerap et al.¹⁰ Polymerase chain reaction (restriction fragment length polymorphism) method was used for allelic determinations at +49. Promoter region

polymorphism was genotyped as follows by using a single primer set: forward, (F) primer: 5-GCT CTA CTT CCT GAA GAC CT-3 and reverse (R) primer: 5-AGT CTC ACT CAC CTT TGC AG-3. PCR was performed on a total volume of 25 μ L containing 1.5 μ L of 1 u/ μ L DNA tag polymerase, 1 μ L of each primer (12.5 picomolar) and 1 μ g of genomic DNA (0.3 μ g/ μ L). gene Amplification was performed with initial denaturation at 94°C (3 min), followed by thirty cycles at 94°C (45 s), 70°C (45 s), 72°C (45 s), and a final extension at 72°C (7 min), resulting in a product of 162 bp. Amplified fragments were digested with BbvI (BsexI) at 65°C for 3 h. Digestion of the amplified 162 bp product with this enzyme resulted in 88 bp and 74 bp fragments. Products were visualized on a 2% agarose gel by using ethidium bromide. A negative (no DNA) control was included in each set of amplifications.

Statistical analysis

The results were compared between the case and control groups according to the age of onset, history of cigarette smoking, tumor size, grade and local or distant metastases. Data were compared using Pearson's chi-square test, Kruskal Wallis and Fisher's exact test. A *P* value of <0.05 was considered statistically significant.

Results

CTLA-4 gene polymorphism at position 49A/G was evaluated in a total of 226 individuals which consisted of 113 patients (85% male) in the case group and 113 healthy individuals (75% male) in the control group. The mean age of the patients was 64.4 \pm 1.2 years and 78 (69%) of them were cigarette smokers. The mean age of patients was 61.64 \pm 4.6 at the time of diagnosis. Table 1 shows the demographic data of the individuals in the case group.

The prevalence of AA, AG and GG genotypes at position 49 of exon-1 according to the PCR-RFLP method was 57.5%, 37.2% and 5.3% in the control group, respectively. While the prevalence of these genotypes in the patient group was: AA in 57.5%, AG in 32.7% and GG in 9.8%. Statistical

analysis using Pearson's chi-square test and a 95% confidence interval revealed no statistically significant difference between the case and control groups (*P*>0.05). There was no significant relationship between these genotypes and the presence of bladder cancer or its stage as compared to the control group (Table 2). There was also no significant difference between the prevalence of the A allele or G allele at this position in both the case and control groups (Table 3).

We also evaluated the possible association of different CTLA-4 genotypes with tumor characteristics including tumor size, mean age of cancer onset and demographic data of the patients as well as risk factors: all of which revealed no association (Table 4).

To demonstrate the possible association of alleles and genotypes at this position with both the stage and invasiveness of the bladder cancer, we compared the presence of different genotypes in low grade, high grade, local and metastatic tumors (Table 5). There were 10 patients (8.8%) who had distant metastases and 30 patients (26.5%) with local metastases after tumor staging.

Out of 10 patients with distant metastases, 6 (60%) had the AA genotype while the others had either the AG or GG genotypes. After statistical analysis of these data, no significant relationship was found between the different genotypes of the CTLA-4 gene at this position and patients' tumor characteristics. Also no association between the age of onset of the disease and different genotypes of the CTLA-4 gene was established. The age at onset of the disease was 61.98, 61.19 and 61.16 in patients with AA, AG and GG genotypes, respectively (*P*=0.97).

Discussion

CTLA-4 is a negative regulator of the B7 family that is the main ligand of CD28. Therefore CTLA-4 inhibits excessive expansion of activated CD28+ T-cells, serving as a negative regulator or feedback inhibitor of the clonal expansion process. There are accumulating data suggesting that CTLA-4 deficiency induces or exacerbates autoimmunity, enhances tumor immunity or

Table 1. Demographic characteristics of patients with bladder cancer.

Demographic characteristics	n=113 (%)
Sex	
• Male	96 (85%)
• Female	17 (15%)
Age (mean±SD)	64.49±1.2
Age of onset of disease (mean±SD)	61.64±2.3
History of smoking	78 (69%)
Tumor characteristics*	
• Low grade tumor	50 (46%)
• High grade tumor	59 (54%)
• Mean tumor size (cm)	4.47
• Distant metastasis	10 (9%)
• Local metastasis	30 (26.5%)
Treatment protocol*	
• TURB**	58 (51.3%)
• Radical cystectomy	8 (7.1%)
• TURB followed by radical cystectomy	47 (41.6%)
• Radiotherapy	9 (8%)
• BCG therapy***	10 (8.8%)
• Chemotherapy	12 (10.6%)

*In these categories, data are combined together from different aspects. Therefore the sum of the data might surpass 100% due to an overlap between data (such as treatment protocols) in the subgroups.**TURB: Transurethral resection of bladder, ***BCG: Bacille-Calmette-Guérin.

prevents induction of immunologic tolerance.¹¹ Stimulated T-cells are suppressed in lymph nodes mainly by transient expression of the CTLA-4 molecule on their own surface after engagement by B7 on APCs. Kato suggested that CTLA-4 engagement promotes T-helper 1 rather than T-helper 2 differentiation.¹²

On the other hand, it is known that immunologic tolerance and anergy allows tumors like bladder cancer to evade the immune response. One of the main treatment protocols for superficial bladder cancer has been the intravesical administration of Bacille-Calmette-Guérin (BCG)

that causes activation of T-cells.^{7,13} Therefore, it can be postulated that factors such as different genetic polymorphisms that lead to suppression or decreased function of T-cells could play a role in tumor genesis in bladder cancer. Li et al investigated the JWA gene promoter polymorphism in 215 patients with bladder cancer and concluded that the -76C allele and 454A allele of this gene were both associated with a significantly increased risk of bladder cancer.¹⁴

Bid et al showed that interleukin-1Ra gene polymorphism was infrequently present in patients with bladder cancer and discussed the important role that interleukin 1Ra gene polymorphism plays in bladder cancer formation.¹⁵

Association of CTLA-4 polymorphism in different types of cancers has been established. Erfani et al. demonstrated that variations in CTLA-4 promoters are important in progression of breast cancer. However, they confirmed that this polymorphism does not play a role in the development of breast cancer.¹⁶ While no association between CTLA-4 polymorphism (AG genotype) and colorectal adenoma could be established in a study involving 132 colorectal cancer and 186 colorectal adenoma patients in an Italian population,¹⁷ another investigation revealed that this type of polymorphism (AG genotype) was actually accompanied by an increased risk of colorectal cancer in the Chinese population.¹⁸ In another study, the role of CTLA-4 polymorphism in increased susceptibility to multiple myeloma was demonstrated.¹⁹

Although association of the CTLA-4 gene with some of the urinary system malignancies such as RCC is well established⁵, according to our knowledge no such study has been performed on bladder cancer until now. Our data showed no correlation between CTLA-4 genotypes at position 49A/G and bladder cancer and no relationship

Table 2. Frequency of different CTLA-4 genotypes in the patients and control groups.

Type of polymorphism	Patient group (n=113)	Control group (n=113)	P value*	Total (n=226)
AA genotype (%)	65 (57.5%)	65 (57.5%)	0.401	130 (57.5%)
AG genotype (%)	42 (37.2%)	37 (32.7%)	0.404	79 (35%)
GG genotype (%)	6 (5.3%)	11 (9.7%)	0.409	17 (7.5%)

*P value of less than 0.05 is considered to be statistically significant using Pearson's chi-square test.

Table 3. Frequency of different alleles of CTLA-4 gene in both patient and control groups.

Groups	"A" allele frequency (%)	"G" allele frequency (%)	P value*
Patient	172 (76.1)	54 (3.9)	0.587
Control	167 (73.9)	59 (26.1)	
Total	339 (75)	113 (25)	

*P value of less than 0.05 is considered to be statistically significant using Pearson's chi-square test.

between the invasiveness of bladder cancer and CTLA-4 gene polymorphism at this position.

It seems in bladder cancer more local factors rather than systemic ones, such as gene polymorphism, might contribute to the pathogenesis. In fact, suppression of T-cell function as a result of CD28 receptor activation on T-cells by CTLA-4 might not be the major mechanism involved in the tumor genesis of bladder cancer. The role of other factors such as defects in humoral immunity, natural killer cell defects and other tumorigenic factors must be investigated.

In conclusion, our data shows no relationship between CTLA-4 polymorphism and genetic susceptibility to the development of bladder cancer and no association with disease progression. Since it is the first study of its kind regarding the polymorphism of CTLA-4 and bladder cancer, therefore additional studies should be performed in both Iranian and other populations in order to have more conclusive results.

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Table 4. Comparison of different CTLA-4 genotypes and their association with tumor characteristics.

Genotype	Age of onset (mean)	Tumor size (mean)
AA genotype	61.98	4.56
AG genotype	61.19	4.20
GG genotype	61.16	5.42
Total	61.64	4.47
P value *	0.935	0.786

*P value of less than 0.05 is considered to be statistically significant using the Kruskal Wallis test.

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Table 5. Comparison of different CTLA-4 genotypes and their association with both grade and disease stage.

Tumor characteristics	AA genotype (%)	AG genotype (%)	GG genotype (%)	P value*	Total (%)
High grade	31 (52.5)	23 (39)	5 (8.5)	0.279	59 (100)
Low grade	31 (62)	18 (36)	1 (2)	0.279	50 (100)
Distant metastasis	6 (60)	4 (40)	-	1.00	10 (100)
Local metastasis	17 (56.7)	6 (20)	7 (23.3)	1.00	30 (100)

*P value of less than 0.05 is considered to be statistically significant using Pearson's chi-square test.

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