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Abstract: Aim: P16, which is localized on chromosome 9p21, is an important tumor suppressor protein of 156 amino acids. The 3'UTR region polymorphisms of the encoding gene has been demonstrated to be related with cancer development and prognosis. MDM2 is an important protein that plays a crucial role in stopping cell cycle at G1/S transition of p53 and preventing apoptosis. According to reports that the T-G base exchange, which occurs at nucleotide 309 of the first intron of the encoding gene, causes increasing in MDM2 RNA and protein levels and inhibition of p53 pathway. The present study focused on investigating the role of p16 (540C>G and 580C>T) and MDM2 (SNP309T>G) gene polymorphisms on the risk and progression of bladder cancer. Materials & Methods: The study consisted of 40 patients diagnosed with bladder cancer and 75 healthy individuals subjects. PCR and RFLP laboratory techniques were used for the polymorphisms. Results: p16 580 C>T genotype distributions were significantly different between patient and control group (p=0.007), but no significant difference was found in terms of p16 540 C> G and MDM2 SNP 309 genotype distributions. p16 580 C>T TT genotype was significantly higher in the patient group than in the control group (p=0.003; OR: 5.625; 95% CI: 1.613-19.614). MDM2 SNP 309 GT genotype was found to be statistically higher than other genotype carriers in our patients with advanced tumor stage (p=0.009; OR: 2.076; 95% CI: 1.184-3.640). In the light of our study’s data, p16 and mdm2-related variants may be an important predictor of bladder cancer susceptibility and disease progression in the Turkish population.

Keywords: p16 540 C > G, p16 580 C>T, MDM2 SNP309, bladder cancer, polymorphism.

1. INTRODUCTION

The incidence of bladder cancer (BC) is increasing and it is the most common urinary malignant tumor worldwide [1]. Various peripheral and genetic risk factors are related with BC development [2, 3] and certain genetic alterations in bladder cancer are considered to be related with cancer susceptibility [4]. Whether genetic polymorphisms has an importance about risk occurrence of bladder cancer remains to be addressed.

Progression of cell proliferation and differentiation is mainly controlled by cyclin proteins, with their pattern of expression increasing and decreasing due to the stage of the cell cycle. Cyclin proteins bind to cyclin-dependent kinases (CDKs), which remain at a constant level during the cell cycle, and stimulate activation of cell-cycle progression. These cyclin and CDKs are characteristic for each phase of the cell cycle and are regulated by cyclin-dependent kinase inhibitors (CKI), which suppress formation of these compounds and inhibit cell proliferation [5, 6]. Tumorigenesis is mainly caused by uncontrolled cell proliferation and delayed differentiation as a result of genetic alterations, polymorphisms and damage in CDK and CKI families [7].

p16 protein is a member of the cyclin dependent kinases inhibitors family, composed of 156 amino acids and encoded by p16 tumor suppressor gene on chromosome 9p21 of the human genome, p16 gene products lead to blocking of G1/S transition by inhibiting the activity of CDK4, CDK6 and cyclin D1 complex which phosphorylates the retinoblastoma (Rb) protein and precipitates the synthesis of transcription factors essential for S phase. Therefore, inactivation of the p16 gene enhances cell proliferation and probably contributes to loss of growth control leading to tumorigenesis [8]. Two polymorphisms of the p16 gene, 540C>G (rs11515) and 580C>T (rs3088440) located in the 3' untranslated region (3'UTR) were recently identified [9]. Several studies showed that these polymorphisms may play a specific role in tumor development, prognosis and aggressiveness by affecting the function of p16 protein [10, 11]. The inhibition of tumor formation is prevented by the p53 tumor suppressor pathway [12].

Somatic mutations that inhibition of the p53 gene have been found in at least half of all human solid
tumors, including BC [13]. MDM2 is a major negative regulator of p53 network, and expression of MDM2 with a high level can related with the inactivation of the p53 pathway, decreasing its tumor suppressor effect and it is located on chromosome 12q14.3- q15 [14,15]. MDM2 gene could be a risk factor in different types of cancer [4]. The T allele showed a lower binding affinity than G allele of MDM2 SNP309 polymorphism to the transcription factor Sp1 which is related with higher levels of MDM2 mRNA and protein in the individuals with G allele, so inhibits the function of p53 pathway in inhibition of tumor formation [12]. There have been a number of studies reporting the association between p16, MDM2 SNP309 polymorphism and bladder cancer risk, but the results were controversial and ambiguous.

Therefore, the present study aimed to investigate the role of p16 (540C>G and 580C>T) and (MDM2) (SNP309T>G) polymorphisms on risk and progression of bladder cancer.

2. MATERIALS AND METHODS

2.1. Participants

The mean ages of patients and control group were (61.62±10.19) and (56.11±13.82) years, respectively. Patients' questionnaires and pathology records were collected from the medical charts to confirm the diagnosis and cancer staging. The control subjects, who were not taking any regular medication by that time, were randomly selected among healthy volunteers. The blood samples were collected after pathological diagnosis prior to any surgical, chemotherapeutic or radiation therapy from those who had not undergone blood transfusion. Stage of the bladder cancers were defined according to the American Joint Committee on Cancer (AJCC) TNM classification. The pathological characterestics for our patients were categorized in T1, T2, T3 and T4 subclasses and G1, G2, G3 tumor grades. Medical Ethics Committee of Haydarpaşa Numune Hospital approval was obtained for the study. The protocol followed was consistent with the World Medical Association Declaration of Helsinki.

2.2. DNA Isolation

Genomic DNA was isolated from the blood leukocytes according to salting-out technique by the method of Miller et al. (1988).

2.3. SNPs Detection

Genotyping was studied by the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). For p16 540 C>G and p16 580 C>T polymorphisms, the following primers were used to amplify; sense primer 5'-GCT CCA ATG TAC GGG GTA AAC-3'; antisense primer 5'-GGGGACTGACTTGGAGTGA CCT-3'. PCR was performed in a final volume of 25 µl with 0.3 µl Taq DNA polymerase (Fermentas, Lithuania). The PCR conditions contained an initial denaturation step as 5 min at 95ºC, followed by 35 cycle of 94ºC 45 sec, annealing at 56ºC for 45 sec and extension 72ºC for 45 sec. The ultimate extension step at 72ºC for 5 min was also studied. For p16 540 C>G, PCR products were digested with MspI (Fermentas, Lithuania) restriction enzyme at 37ºC for 16 h and visualized on 3% agarose gels and stained with ethidium bromide. Genotypes were defined as GG (181 bp), CG (181,104,77 bp), CC (104,77 bp) for p16. For p16 580 C>T polymorphism the products of PCR were digested with HaeIII (Fermentas, Lithuania) restriction enzyme at 37ºC for 16 h and visualized on 3% agarose gels and stained with ethidium bromide. Genotypes were determined as TT (181 bp), CT (181,142,39 bp), CC (142,39 bp). The oligonucleotide primers to amplify region of MDM2 SNP309 were: 5'-CGG TCA GGG TAA AGG AGT TAA C-3' (sense), 5'-AGC TGG AGA CAA GTC AGG ACT CAA C-3' (antisense) respectively. The PCR mix was incubated for 5 min at 95ºC followed by 35 cycles of 45 sec at 94ºC, 45 sec at 56ºC, 72ºC for 45 sec.

The final step at 72ºC for 5 min was also studied for MDM2 SNP309 polymorphism. PCR products were digested with MSPA1I (Fermentas, Lithuania) restriction enzyme at 37ºC for 16 h and visualized by electrophoresis in a 3% agarose gel. Genotypes were determined as TT (237 bp), GG (189, 48 bp) or GT (237, 189, 48 bp) for MDM2 SNP309 polymorphism.

2.4. Statistical Analysis

Clinical parameters and demographic characteristics are expressed as mean±SD, frequency, and the percentage. The statistical data was analysed with Statistical Package for the Social Sciences (SPSS) software (version 20; IBM, SPSS Inc, NY, USA) and Chi-Square test. Student's t-test and ANOVA were applied if required. The estimated odds ratios (OR) with 95% confidence interval (CI) were determined to
evaluate potential risk. Values were considered as statistically significant if the two-tailed p-value was less than 0.05.

2.5. Characteristics of Subjects

For this study, it is determined p16 (540C>G and 580C>T) and MDM2 (SNP309T>G) gene polymorphisms in 40 patients and 75 controls. All the study groups (patients and control group) had similar distributions of the age, gender, family history, smoking and alcohol consumption. All of the patients had smoking history. There was no remarkable age difference among the cases and the controls. The some tumor characteristics of our patients are given in Table 1.

3. RESULTS

Table 2 shows the genotypes frequencies for p16 540 C>G, p16 580 C>T and MDM2 SNP309

![Figure 1: RFLP agarose gel pattern of p16 580, p16 540 and MDM2 polymorphisms.](image)

Table 1: Tumor Characteristics of our Bladder Cancer Patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Bladder Cancer Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Tumor Stage (n=38) (2 patients pathological data is unavailable)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>23</td>
</tr>
<tr>
<td>T2</td>
<td>9</td>
</tr>
<tr>
<td>T3</td>
<td>5</td>
</tr>
<tr>
<td>T4</td>
<td>1</td>
</tr>
<tr>
<td>Tumor Grade (n=38) (2 patients pathological data is unavailable)</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>23</td>
</tr>
<tr>
<td>G2</td>
<td>9</td>
</tr>
<tr>
<td>G3</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2: Genotype Frequencies of p16 and mdm2 Gene Variants for Our Study Groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>p16 580C&gt;T Patient n=40 (%)</th>
<th>Control n=75 (%)</th>
<th>p16 540C&gt;G Patient n=40 (%)</th>
<th>Control n=75 (%)</th>
<th>MDM2 309T&gt;G Patient n=40 (%)</th>
<th>Control n=75 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>25(62.5)</td>
<td>54(72)</td>
<td>21(52.5)</td>
<td>26(34.7)</td>
<td>5(12.5)</td>
<td>15(20)</td>
</tr>
<tr>
<td>CT</td>
<td>6(15.0)</td>
<td>18(24.0)</td>
<td>17(42.5)</td>
<td>36(48)</td>
<td>23(57.5)</td>
<td>37(49.3)</td>
</tr>
<tr>
<td>TT</td>
<td>9(22.5)</td>
<td>3(4.0)</td>
<td>2(5)</td>
<td>13(17.3)</td>
<td>12(30)</td>
<td>23(30.7)</td>
</tr>
</tbody>
</table>

P=0.007 P > 0.05 P > 0.05
According to our results, there was a statistically significant difference between patients and controls for the distribution of p16 580 C>T genotype (p=0.007). There was no significant difference among patients and controls for the distribution of p16 540 C>G or MDM2 SNP 309 genotypes and alleles. The frequency of p16 580 C>T TT genotype was significantly increased in bladder cancer patients compared to those with controls (p=0.003; OR: 5.625; 95% CI: 1.613-19.614).

The distribution of p16 and MDM2 gene variants according to tumor characteristics are present in Table 3. MDM2 SNP 309 TG genotype was significantly increased in our patient group with advanced tumor staging compared to those with early stage tumors (p=0.009; OR: 2.076; 95% CI: 1.184-3.640).

<table>
<thead>
<tr>
<th>Tumor Pathology Parameters</th>
<th>P16 580 C&gt;T</th>
<th>P16 540 C&gt;G</th>
<th>MDM2 309 T&gt;G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC  CT  TT</td>
<td>CC  CG  GG</td>
<td>TT  TG  GG</td>
</tr>
<tr>
<td>Patients with advanced tumor stage (T3+T4)</td>
<td>n</td>
<td>4  1  1</td>
<td>2  4  0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>66.7 16.7 16.7</td>
<td>33.3 66.7 0</td>
</tr>
<tr>
<td>Patients with early tumor stage (T1+T2)</td>
<td>n</td>
<td>20  5  7</td>
<td>17  13  2</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>62.5 15.6 21.9</td>
<td>53.1 40.6 6.2</td>
</tr>
<tr>
<td>High grade tumor (G3)</td>
<td>n</td>
<td>4  1  1</td>
<td>2  4  0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>66.7 16.7 16.7</td>
<td>33.3 66.7 0</td>
</tr>
<tr>
<td>Low Grade tumor (G1+G2)</td>
<td>n</td>
<td>20  5  7</td>
<td>17  13  2</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>62.5 15.6 21.9</td>
<td>53.1 40.6 6.2</td>
</tr>
<tr>
<td>Low grade tumor (G1)</td>
<td>n</td>
<td>14  4  5</td>
<td>10  11  2</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>60.9 17.4 21.7</td>
<td>43.5 47.8 8.7</td>
</tr>
<tr>
<td>High Grade tumor (G2+G3)</td>
<td>n</td>
<td>10  2  3</td>
<td>9  6  0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>66.7 13.3 20</td>
<td>60  40  0</td>
</tr>
</tbody>
</table>

It was recently understood that polymorphism studies provide very important information about growth of cancer and progression besides toxicity of anticancer drugs and the performance of cancer treatment [19]. The p16 protein, which has an important role in the cell cycle process, was a specific inhibitor of CDK4 and CDK6 and was also referred as a tumor suppressor gene in most malignancies. It was known that the loss of function in p16 results in the inactivation of its growth suppressor effect [20]. Cell proliferation increases by the inactivation of the p16 gene, possibly contributes to loss of growth control leading to tumorigenesis [8]. Two polymorphisms of the p16 gene, rs11515 and rs3088440 located in the 3’untranslated region (3’UTR) were recently identified [9]. Recent study implied that p16 gene polymorphisms were thought to be an important factor in tumor development, prognosis and aggressiveness by affecting the function of p16 protein [10, 11]. Accordingly, in the present study, the evaluation of the possible association of p16 540 C>G and p16 580 C>T polymorphisms with the clinical and pathological parameters besides the development of BC was aimed.

### 4. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

### 5. DISCUSSION

Cancer is a major public health problem all over the world, and it is one of the primary causes of morbidity and mortality [16]. The etiology of cancer is complex and is still obscure. Recent research suggests that SNP in genes play critical roles in cancer development and progression [17, 18].

In the study of Nakazawa et al. p16 expression was evaluated in urine cytological and histological samples, and the study reported that a high incidence of p16 overexpression in high-grade urothelial carcinoma was noted in cytological samples and that immunocytochemical analysis of p16 is a useful method for detecting urothelial carcinoma and the tumor
infiltrating potential [21]. The genetic alteration of the p16 and p14 genes in BC researched by the investigators in another study, and they did not find any association between tumor grade/stage and p16 alterations. However, the deletion of the p14 gene was mostly observed in poorly differentiated tumors. This study also noted that p16 plays a role in early tumorigenesis [22]. Conversely, Deniz et al. reported that the p16 gene was more commonly changed in patients with high grade bladder cancer. Krüger et al. observed the prognostic effect of p16 alterations in patients with T1 stage BC and concluded that there is a remarkable correlation among the case of p16 and progression-free survival. But, they did not find any significant correlations between p16 status and the tumor grade [23].

Kumar et al. reported the prevalence of the heterozygous genotype of CDKN2 p16 540C>G as 29% the population of Swedish and in similar populations, it was reported as 22% [24]. In addition, a 26% prevalence of the CDKN2 p16 580C>T heterozygous genotype was reported in the Swedish population and this was shown in accordance with Australians [24, 25]. In the present report, the genotype distribution of CDKN2 p16 540 C>G was 48% CG 34.7% CC, and 17.3% GG. The prevalence of the heterozygous genotype of CDKN2 p16 580 C>T was 24%, which was in accordance with the previous reports of Kumar (Kumar et al.) and Holland (Holland et al.).

Chen et al. found an association between p16 540 C>G or p16 580 C>T genotypes and pancreatic cancer as well as the study by Zheng et al. performed in head and neck cancer [26, 27]. In the present report, there was a statistically significant difference between patients and controls for the distribution p16 580 C>T genotype. The frequency of p16 580 C>T TT genotype was significantly increased in bladder cancer patients compared to those with controls. On the other hand, we did not observe any association between BC and p16 540 C>T gene polymorphisms. p53 tumor suppressor pathway plays an important role in the prevention of tumor formation, and MDM2 is mainly a negative regulator of p53. The overexpression of MDM2 can decrease the level of p53 protein and eventually results in the dysfunction of the p53 pathway [12]. Bond et al. reported that the transcription of the MDM2 gene can be increased by the high binding affinity of G allele to the Sp1 transcriptional activator in MDM2 polymorphism. Therefore, MDM2 SNP309 T>G polymorphism is related with tumor formation and adverse clinical behaviors of tumors, such as fast progression and poor treatment response [28].

Some studies found that G allele of MDM2 SNP309 T>G polymorphism was related with increased risk of some tumor, such as leukemia, gastric cancer, hepatocellular cancer, and colorectal cancer [29-32]. However, inverted results were observed in some other tumors, for example, head and neck cancer and prostate cancer [33, 34] and studies suggested that the protective role of G allele may exist in these tumors. Therefore, the formation of various type of cancer might be related with this SNP that we studied. The difference might be influenced by different sample size and different genetic background [35].

There are some disagreement about the relation between MDM2 SNP309 T>G polymorphism and bladder cancer risk according to studies that published before. Studies by Wang et al., Horikawa et al. and Hitzenbichler et al. showed no remarkable association between the MDM2 SNP309 T>G polymorphism and BC risk [36-38] Gangwar and Mittal suggested that MDM2 SNP309 T>G polymorphism may decrease formation of breast cancer, whereas Onat et al. showed that increasing risk of BC might be associated by the MDM2 polymorphisms [39, 40]. In our study based on 40 cases and 75 controls, no significant association was observed between MDM2 SNP309 T>G polymorphism and bladder cancer risk. However, in the stratification analysis between the MDM2 SNP309 T>G polymorphism and bladder cancer status, we found that MDM2 SNP 309 TG genotype was significantly increased in our patient group with advanced tumor staging compared to those with early stage tumors. This result implied that SNP309 TG genotypes might be associated with the progression, but not on the risk of our bladder cancer patients.

Consequently, the present study was a preliminary study to establish the link between CDKN2 p16 540 C>G, CDKN2 p16 580 C>T, and MDM2 SNP309 T>G polymorphisms and pathogenesis of BC among our patients. The number of bladder cancer patients is the limitation of our study. This may be increased for collaborating with other Universities or Research Centers in the future for Turkish population. Additional studies with larger sample sizes are needed to define the influence of CDKN2 p16 540 C>G, CDKN2 p16 580 C>T, and MDM2 SNP309 T>G genotyping on
clinical outcomes. The authors believe that the results of the present study could be more conclusive with further studies, which determines the interaction of p16 and MDM2 gene.

REFERENCES


