Predictive value of p53 and NAT2 enzyme for disease free survival in patients with superficial bladder cancer

AHMET ERBAĞÇI¹, FARUK YAĞCI¹, KEMAL SARICA¹, METİN KARAKÖK², SAKIP ERTURHAN¹

Departments of ¹Urology and ²Pathology, Şahinbey Hospital, Gaziantep University Medical School, Gaziantep-Turkey

Considering the higher recurrence rates in superficial bladder cancer, p53 immunoreactivity together with functional differences of N-acetytransferase-2 (NAT2) enzymes were studied to evaluate their overall effects on disease free survival rates (DFS) in 45 patients with superficial bladder cancer. Between 1994 and 2001, 45 patients with bladder cancer have been evaluated. All patients underwent transurethral resection of the tumor with or without intracavitary treatment. During follow-up period, ranging from 7 to 122 months (mean 27.3) DFS rates were comparatively evaluated with p53 immunoreactivity in tissue specimens obtained together with the functional differences of NAT2 assessed serologically. Immunohistochemical (IHC) study was used to evaluate p53 immunoreactivity. NAT2 alleles were differentiated by polymerase chain reaction using the restriction fragment length polymorphism (PCR-RFLP). According to our results, the overall p53 immunoreactivity was 35.5%. As a sole parameter, p53 immunoreactivity of bladder cancer did not affect DFS (p>0.05). P53 immunoreactivity classified with tumor grade according to WHO criteria were as follows; 29% of low malignant potential, 38% of low-grade cancer and 29% of high grade of cancer. Tumor grade in p53 (+) patients was the only parameter showing differences in the DFS in low grade bladder cancer (p<0.05). According to functionally different subtypes of NAT2; slow type NAT2 were 55% (n: 25) and rapid type NAT2 were 45% (n: 20). Patients showing p53 negativity and rapid subtype of NAT2 enzyme exhibited approximately two fold differences in DFS. In our present study, although the patient number was limited, we were able to show long-term DFS in case of rapid type NAT2 and p53 negativity. P53 positivity and slow type NAT2 might be accepted as a bad prognostic factor for DFS in superficial bladder cancers. [Turk J Cancer 2002;32(3):105-115]

Key words: Transitional cell carcinoma, p53, N-acetyltransferase (NAT), surveillance
Bladder cancer is estimated as the fourth most common cause of cancer death in men, which comprises 90% of all bladder cancers and is classified histopathologically into superficial, infiltrating and invasive according to the TNM guidelines (1,2). Prognosis is greatly worsened by muscle-invasive disease at presentation and over half of those with superficial disease will develop recurrence (1). Thus, related with progression of the disease, the ultimate goal for many of the studies is to help predict which superficial tumor will progress to muscle invasion (10-25% of tumors) allowing possible early aggressive intervention in this group. Therefore, it seems interesting to determine the phenotypic properties of tumor cells, which would be useful to determine the prognosis of a malignant tumor by trying to develop the simplest and most reproducible methods.

Among the various factors evaluated in this aspect, biomarkers signaling for tumor onset, waste/end products of tumor metabolism, tumor ploidy, oncogenes, antioncogenes, growth factors, tumor associated antigens, proliferation associated proteins such as Ki67 and more recently p53 mutations have been extensively studied (3-8). P53 is a tumor suppressor gene and the presence of a protein p53 mutation can contribute to tumor growth. Several studies have shown that p53 expression is associated with grade and stage; but also with tumor progression (9-13). However, unfortunately some literature data have been including normal type of p53 accumulation at nucleus (wild form) may be possible to be assessed immunohistochemically because of some inductive factors such as BCG immunotherapy (14,15).

In another point, NAT2 is involved in acetylating of aryl amines notably from occupational exposure and smoking which has been implicated in the metabolic activation of these substances to ultimate carcinogens. Polymorphism of NAT2 accounts for the acetylating phenotype either rapid or slow, the latter established as a susceptibility factor for the development of cancer and slow acetylating form accused for bladder cancer (16-19).

In this present study, our aim was to evaluate the DFS of superficial bladder cancer, its relation with immunohistochemical p53 positivity together with functional difference of NAT2 enzyme. DFS analysis may help to find a clue on recurrences of superficial bladder cancer according to p53 immunoreactivity state and different functional state of NAT2 (20).

Materials and Methods

Forty-five patients (7 female, 38 male) aging from 34 to 87 years (mean 63) were enrolled in this study and in addition to a detailed urological anamnesis, physical and radiological examination including conventional ultrasonography, cystography of bladder have been performed. Intravenous pyelography, computerised tomography (CT), magnetic resonance imaging (MRI) and bone scintigraphy examinations were applied when needed for staging or in case of suspicion of upper urinary system malfunction. Following resection of the superficial bladder tumor, patients' clinical characteristics were evaluated comparatively with p53 immunostaining state and different functional subtype of NAT2 (Table 1). Follow-up period was 7-122 months (mean 27.3).
Table 1
Comparative evaluation of the histological classification, functional differences of NAT2 enzymes and p53 positivity

<table>
<thead>
<tr>
<th></th>
<th>p53</th>
<th>p53</th>
<th>N-acetyltransferase-2 (NAT2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>(%)</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low malignant potential</td>
<td>7</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Low grade Ca</td>
<td>21</td>
<td>47</td>
<td>13</td>
</tr>
<tr>
<td>High grade Ca</td>
<td>17</td>
<td>37</td>
<td>12</td>
</tr>
<tr>
<td>NAT2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow acetylator</td>
<td>25</td>
<td>55</td>
<td>16</td>
</tr>
<tr>
<td>Rapid acetylator</td>
<td>20</td>
<td>45</td>
<td>18</td>
</tr>
</tbody>
</table>

Specimens were obtained by cold cup biopsy and/or transurethral resection of bladder tumor in 45 patients. Tumor mitotic index and necrosis were histologically searched with hematoxyline eosin (H&E) stained preparation and classified according to WHO criteria. Tumor grade was evaluated with new criteria according to new classification systems of WHO, 1998 meeting (21).

P53 accumulation was evaluated with immunohistochemical (IHC) methods described below:

**Immunostaining procedure:** The tumor blocks with the highest tumor grade were selected for immunostaining. The paraffin sections were put on adhesive slides prepared with poly-L-lysine and heated up to 60°C during 2 hours in overnight two days. The sections were de-waxed in xylene, rehydrated in a series of graded alcohol's and treated for 10 minutes with 0.5% peroxidase in methanol to block endogenous peroxidase. The rehydrated slides were placed in a plastic coupling jar filled with antigen retrieval Citra solution. Three coupling jars were incubated simultaneously in a microwave oven for 15 minutes at 500 watts. After washing in tris buffer saline (pH 7.6) the sections were incubated for 10 minutes with nonreactive goat serum.

The prediluted monoclonal antibody DO-7 was used for p53 immunostaining and the sections were incubated for 30 minutes at room temperature. The immunoperoxidase technique used to stain the sections was a streptavidin, biotin and peroxidase detection system (supersensitive HRP, horse-radish-peroxidase).

**Immunohistochemical staining (IHC):** Immunostaining was done by monoclonal antibody DO-7 (Antigen Retrieval Citra, Biogenex) solution and supersensitive HRP (Biogenex), slides were evaluated by experts who had no knowledge of the clinical data or treatment outcome. The entire slide was examined and for each field at x200 magnification, 0.57-mm² areas with few exceptions greater than 500 cells per field, the percentage of tumor cells with positive nuclear staining was evaluated. The field with the highest percentage of
tumor cells with nuclear staining was regarded as representative for the tumor, which was considered to have altered or unaltered immunohistochemical staining.

Immunoreactions were evaluated as positive for p53 overexpression when more than 1% neoplastic cells contained the reaction product in the nucleus. Cytoplasmic coloration had no means being related with p53 accumulation, but results exactly have been accepted as negative only when no nuclear coloration occurred.

Identification of NAT2 mutation and genotype: At the latest cross-sectional evaluation on 4th January 2001, 10 ml venous blood samples derived from each subjects were drawn in ETDA and stored at -20°C until transported to processing. DNA were extracted from leukocytes manually by standard three-step phenol/chloroform extraction and stored at +4°C until further analysis. The 1211 base pair fragments (bp) containing the coding region of the NAT2 gene were amplified by polymerase chain reaction. Seven-mutation site were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and alleles were classified according to literature as well (17).

Fisher’s Exact Test for matched groups was used to compare p53 overexpression between each group. For survival comparison long-rank test and Kaplan-Meier methods were used. P <0.05 was accepted as significance level and SPSS software programme was used for the statistical analysis.

Results

Of the 45 patients evaluated, clinical analysis and functional difference of NAT2 versus p53 immunoreactive staining state were presented at table 1. Given a cut-off point for tumor positivity of more than 1% of immunostained nuclei representing any mutant type p53, the overall p53 immunoreactivity was 35.5% in all cases (29 of p53 (-), 16 of p53 (+)). DFS durations were 65.5±11.4 months in p53 (-) patients and 34.4±4.2 months in p53 (+) patients (p>0.05), respectively (Figure 1). Demographic data and NAT2 genotype and its relation with clinical state were presented at table 2. When different acetylating subtype of NAT2 in relation with bladder tumor occurrence was evaluated, we found 20 (45%) patients with rapid acetylating enzymes and 25 (55%) patients with slow acetylating enzymes. DFS were calculated as 58±11.4 months in slow acetylating and 78±21.2 months in rapid acetylating types, respectively.

Amongst 45 patients, 16% was found to have low malignant potential cancer, 47% in low grade cancer and 37% in high grade cancer. DFS according to grade and p53 immunoreactivity is presented in figures 2 and 3. Histological evaluation and distribution of slow acetylating status showed 57% of low malignant potential cancer, 52% of low grade cancer and 59% of high grade cancer, respectively. In point of DFS, p53 (-) patients showed statistically significant difference between all grades (p<0.05). But same relation was not found in p53 (+) patients. There were not any difference between grades and distribution of slow and rapid acetylating state (p>0.05).

DFS in p53 (-)/rapid acetylating group (low risk group) was 118±13.3 months and in p53 (+)/rapid acetylating group was 42±14.3 months, respectively (Figures 4 and 5). DFS according to slow acetylating state were 71.3±14.5 months in
p53 (-)/slow acetylator and 37.5±9.5 months in p53 (+)/slow acetylator group (high risk group).

Table 2
Evaluation of NAT2 genotype and its clinical presentation in our group

<table>
<thead>
<tr>
<th>NAT2 Genotype</th>
<th>Slow acetylating phenotype</th>
<th>Rapid acetylating phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>5B*/5B*</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>5B*/5C*</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>5B*/6A*</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>5C*/6A*</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>6A*/6A*</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Total (n: 45)</td>
<td>(n: 25)</td>
<td>55</td>
</tr>
</tbody>
</table>

Fig 1. Comparative evaluation of DFS duration with respect to p53 immunoreactivity
**Fig 2.** Separate evaluation of the effect of p53 negativity on DFS rates with an emphasis on the grade of the tumors.

**Fig 3.** Separate evaluation of the effect of p53 positivity on DFS rates with an emphasis on the grade of the tumors.
Fig 4. Comparative evaluation of the effect of p53 immunoreactivity in combination with slow type NAT2.

Fig 5. Comparative evaluation of the effect of p53 immunoreactivity in combination with rapid type NAT2.
Discussion

Recent researches have focussed on combining pathological variables with molecular markers to identify the phenotypes likely to progress as well as contributing to the overall transcriptional fingerprint of urothelial cancer (13). The partial interest among the parameters turned to mutant form of the p53 and functional differences of detoxification/acetylating (with onset, end or side products) in metabolism together with certain risk conditions have been taken place in the research armamentarium nowadays. Of these factors evaluated, originally discovered in SV 40-transformed tumor cells, the p53 gene is now regarded as a major tumor suppressor gene and the results of some relevant studies have demonstrated it to be involved in a trigger mechanism of not only bladder tumor development, but also other malignancies like prostate and renal tumor (19,22). However, mutations in the gene may result in an altered protein that has lost its suppressive regulatory effect (23-25). But, no matter how many gene mutation of p53 occurred, immunohistochemical staining is the best indicator for p53 protein accumulation around the nucleus (7,8).

Regarding the predictive value of p53 positivity and related proteins; there are contradictory data reported in the literature and several studies with larger groups of patients were failed to find any role of p53 IHC as an independent prognostic marker in bladder cancer when using polyclonal antibodies against p53 (26). But some authors found that p53 positivity predicted time to recurrence in a univariate analysis in superficial tumors (27). Again in another study dealing with superficial disease, p53-positive IHC has been found to be an independent prognostic marker for progression of Ta bladder cancer (28). However, contradictory results regarding the predictive value of this parameter made it hard to accept p53 as a sole prognostic marker in bladder cancer patients (20,29-31).

In our study, of the 45 patients with recurrent bladder cancer, 29 have shown p53 negativity and the remaining 16 had p53 positivity, thus the overall p53 immunoreactivity was 35.5% in all cases and that was similar with the data derived from literature. In survival analysis, while p53 (-) patients showed 65.5±11.4 months duration of DFS, in p53 (+) patients the duration has been found to be 34.4±4.2 months, which was approximately two-fold high compared with the p53 (-) patients.

Despite the non-homogenous distribution of bladder cancer patients with histological grading, p53 (+) staining with respect to grade of the tumors, has been found to be 29% in low malignant potential cancers, 38% in low grade cancers and 29% in high grade cancers, respectively. On the other hand, the percentage of p53 negativity in various grades of cancer has been found to be statistically different from each other in our group (p<0.05). Unfortunately, we were not able to show the same difference with respect to p53 positivity (p>0.05).

NAT2 is involved in acetylating of aryl amines notably from occupational exposure and smoking, which has been implicated in the metabolic activation of these substances to ultimate carcinogens. Polymorphism of NAT2 accounts for the acetylating phenotype either rapid or slow, the latter established as a susceptibility factor for bladder cancer (18,19).

Taking the contradictory results reported for the predictive value of p53 positivity in patients with bladder cancer and the possible additive predictive value of NAT2 into account, some certain types of cancers have shown frequently
either slow or rapid type of NAT2 activity. The role of different type of acetylating state of NAT2 on p53 mutation may have hypothetically an explanation. The chromosomal location of both NAT1 and NAT2 is on chromosome 8p21.3-23.1. This lies close to, or possibly within a region shown to be deleted in certain invasive bladder tumors (18). Regardless of any relation of these two factors, different functional type of NAT2 and its relation with p53 staining on bladder cancer may play trigger role as promoter in this manner. We proposed this prospective study in an attempt to outline the predictive value of both parameters for the DFS in patients with bladder cancer.

Functional difference of NAT enzyme has been comparatively evaluated with p53 positivity in our patients to predict its effect on DFS. The distribution of NAT2 enzymes in 45 patients was 20 (45%) of rapid acetylating and 25 (55%) of slow acetylating. DFS were found 58±11.4 months in slow acetylating group and 78±21.2 months in rapid acetylating group. The slow acetylating status of NAT2 enzyme according to grades were 57% low malignant potential cancers, 52% low grade cancer and 59% high grade cancer. In relation with slow acetylating NAT2 enzyme, grade of bladder cancer did not show statistically significant difference (p>0.05). We did not find a particular slow or rapid allele. But like the literature data, we found a few alleles related with slow and rapid acetylating phenotype (18). Again comparing with 39.3% frequencies of slow type NAT2 enzymes in normal population, we found 55% of slow type NAT2 enzymes in recurrent bladder tumor, comparable with the previous knowledge (17,18). NAT2 allele and related phenotype have been reported to vary in population but the functional difference of NAT2 enzyme is not also sole prognostic marker for bladder tumor (16,18). These two parameters (p53 immunoreactivity and functional types of NAT2) and their relation with prognosis of the bladder cancer may be also expressed by using DFS durations.

Overall DFS were 118±13.3 months and 42±14.3 months in p53 (-)/rapid acetylating group and p53 (+)/rapid acetylating group, respectively. DFS according to slow acetylating state were 71.3±14.5 months in p53 (-)/slow acetylating and 37.5±9.5 months in p53 (+)/slow acetylating group. Comparative evaluation of the results demonstrated that tumor recurrence has been found to be at least two fold different by means of DFS (p<0.05). Patients with p53 (+)/slow acetylating NAT2 enzyme has the worst DFS compared with other risk factors. Conversely p53 (-)/rapid acetylating NAT2 enzymes have the longest DFS.

In conclusion, among the predictive parameters related with the tumor recurrence in patients with superficial bladder cancer, particular parameters with varying prognostic values have been reported. P53 tumor suppressor gene has been studied extensively in recent years. However, functionally different type of NAT2 enzyme predicting behaviour of superficial bladder cancer has been reported to have conflicting results. In our present prospective study, although the patient number was limited, we were able to show long-term DFS in rapid type NAT2 and that of p53 negativity. Moreover, our findings indicated that p53 positivity together with slow type NAT2 might be accepted as a bad prognostic factor for DFS in superficial bladder cancer.
References


