Evaluation of Retinoblastoma Gene Product (RB) as A Prognostic Indicator in Bladder Carcinoma

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ABSTRACT

Background: Despite precise pathologic staging and grading, we are unable to predict clinical outcome in patients with bladder cancer. The retinoblastoma susceptibility gene (RB) a prototype of tumour suppressor genes, thought to function as a cell cycle regulator, has recently been associated with development and/or progression of bladder cancer, as well as sarcoma and small cell lung cancer.

Purpose: The aim of this study was to investigate the hypothesis that altered patterns of RB expression correlate with prognosis and with tumour cell growth fraction in bladder cancer.

Methods: Tumours from 40 patients with primary transitional cell carcinoma were studied. Expression of RB gene product was assessed immunohistochemically. The proportion of cells expressing the ki67 antigen was also detected.

Results: Three patterns of RB protein staining were observed, absent n = 12, heterogenous n = 23 and strongly homogenous n = 5. Cases with loss of RB protein and those with apparent over expression were considered to have an altered RB protein expression. Altered expression of RB was significantly associated with muscle invasive growth and high tumour grade (p < 0.0001, p < 0.001) respectively. The 5 years survival rate was significantly decreased in patients with altered RB protein expression compared with patients with normal expression of RB (p < 0.05). There were significant differences in the ki67 index between poorly (G3) and better (G1/G2) differentiated tumours and between invasive and non muscle invasive tumours.

Conclusion: This study suggests that altered patterns of RB protein expression may be an important prognostic variable in patients presenting with invasive bladder cancer.

Key Words: Retinoblastoma gene product - Bladder carcinoma - Prognosis.

INTRODUCTION

The existence of tumour suppressor genes has been suggested by early experiments where fusion of tumour cells with normal cells leads to a suppression of the neoplastic phenotype in the formed hybrid cells. The retinoblastoma gene (RB) named after the eye tumour retinoblastoma that is a childhood cancer of embryonal retinoblasts. RB mutation is responsible for the retinoblastoma, however survivingly patients are particularly prone to develop a second primary tumour, particularly osteosarcoma, small cell lung carcinoma, breast carcinoma and genitourinary carcinomas [6]. Introduction of RB gene into RB negative bladder cancer lines resulted in tumour suppression [7]. RB gene product can be detected using immunohistochemical methods. Cellular levels of RB protein vary during the cell cycle, but complete absence of staining throughout a tumour is indicative of RB gene alteration [19]. In this study the expression of RB protein was assessed by immunohistochemistry and it was correlated with 5 years survival and with tumour growth fraction.

PATIENTS AND METHODS

Tumours from 40 patients were selected from cases admitted to urology Department of Ain Shams University Hospital between 1995-2000. Tumour samples were taken by means of cystoscopic resection (n = 25) and cystectomy (n = 15). Tumours were staged by examination under anaesthesia and on the basis of histological examination according to the absence (Pta) or presence (T1) of invasion of the lamina propria or invasion of the detruser muscle (T2-T4) (UICC system). Tumour grade was assessed by examination of paraffin sections.

Immunostaining procedure:

Tumour blocks with the highest tumour grade with maximal invasion were selected for
immunostaining. 5 µm sections were cut and mounted on slides. The sections were dewaxed in xylene, rehydrated in a series of graded alcohol and treated for 10 minutes with 0.5% peroxidase in methanol to block endogenous peroxidase. To expose the antigenicity of tissue, slides were incubated in a microwave oven for 40 minutes at 650 watts.

Immunostaining for retinoblastoma gene product (RB) was performed with the monoclonal mouse anti-human antibody. For measurement of tumour growth fraction, sections were incubated with ki67 antibody. Streptavidin-biotin complex immunoperoxidase method (ABC) was applied. The peroxidase reaction was developed using diaminobenzidine as a chromagen and sections were counterstained with haematoxylin. Appropriate positive control sections were included with each staining run (a known RB positive breast carcinoma). Each run also included negative controls, prepared by staining duplicate section of every tumour using the methods described above, but omitting the primary antibody.

**Evaluation for immunostaining:**

A tumour with an altered RB expression was defined as a tumour with no nuclear staining of tumour cells, in contrast to positively stained mesenchymal cells in the same area. Strongly homogenous nuclear staining in the majority of tumour cells was also considered to be an altered RB expression while heterogenous staining was considered normal [8].

To obtain a measure of tumour growth fraction, random fields were selected in well preserved areas of each section and tumour cells were counted to determine the proportion of cells showing any nuclear reactivity (ki67 index, expressed as a percentage). At least 500 cells were assessed for each tumour.

**Statistical analysis:**

Differences in the distribution of variables between different groups were tested with chi-square or students t-test. Overall survival curves for RB expression was calculated according to the method of Kaplan and Meier and the degree of significance was determined by the log-rank test.

**RESULTS**

This study included 40 samples of primary bladder carcinoma classified histologically as transitional cell carcinoma (TCC) (n = 33), TCC with squamous differentiation (n = 5) and TCC with glandular differentiation (n = 2). 30 patients were males and 10 patients were females. The mean age was 53 (range 36-73). Table (2) shows staging and histopathologic grading of the tumours.

**RB expression:**

Three patterns of RB nuclear protein staining were observed absent (n = 12), strongly homogenous n = 5 and heterogenous staining (n = 23) (57.5%), cases with loss of RB protein and those with apparent over expression (n = 17) (42.5%) were considered to have an altered RB expression (Figs. 2-5).

Altered RB expression was significantly associated with muscle invasive growth (p < 0.0001) and high tumour grade (p < 0.001) (Table 1). Of the 17 cases with altered RB expression 5 cases (29.4%) were included in the well to moderately differentiated (G1/G2) tumours while 12 cases (70.6%) were included in the poorly differentiated (G3) tumours.

Also, of the 17 cases with altered RB expression 4 cases (23.5%) were superficial non invasive while 13 cases (76.5%) were included in the muscle invasive group. However, within the superficial and muscle invasive groups, no difference between individual subgroups with respect to RB protein expression, could be detected.

The five years survival was significantly decreased in patients with altered RB expression compared with survival in patients with normal expression (p < 0.05) (Fig. 1).

**Tumour ki67 index (growth fraction), association with tumour stage, grade and RB expression:**

The ki67 index was assessed in 40 cases and showed a very wide range from 1 to 48.3% (Table 2) (Fig. 6). There were significant differences in the ki67 index between poorly (G3) and better (G1/G2) differentiated tumours (mean 19% and 6.5%) respectively and between invasive and non invasive tumours (mean 18.5% and 7%) respectively. Ki67 indices for tumours with altered RB protein were significantly greater than those showing normal RB expression (p < 0.05) (Table 2).
Fig. (1): Kaplan-Meier survival curve.

Fig. (2): Transitional cell carcinoma of the bladder (superficial) showing expression of RB protein. Labeling of tumour cell nuclei is of variable intensity (x250) (ABC technique) (DAB-Haematoxylin counter stain).

Fig. (3): Transitional cell carcinoma of the bladder (grade 3), showing homogenous expression of RB protein in tumour cell nuclei (x100) (ABC technique) (DAB-Haematoxylin counter stain).

Fig. (4): Transitional cell carcinoma of the bladder with glandular differentiation showing variable expression of RB protein in tumour cell nuclei (x100) (ABC technique) (DAB-Haematoxylin counter stain).

Fig. (5): Transitional cell carcinoma of bladder showing absence of immunohistochemical staining for RB protein (ABC technique) (x100) (DAB-Haematoxylin counter stain).

Fig. (6): Ki-67 immunostaining of transitional cell carcinoma with squamous differentiation showing high proliferative activity (ABC technique (X100) (DAB-Haematoxylin counter stain).
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scored nine (19%) as negative for RB expression (also assessed immunohistochemically using frozen sections), although their negative group also included tumours showing < 10% positive cells. Both studies may underestimate the proportion of tumours with alterations in the RB gene, as mutations do not necessarily lead to diminished antigenicity. Cairns et al. [3] found a loss of heterozygosity (LOH) at the RB locus in 30% (28 of 94 tumours from informative patients), but it remains possible that at least in some bladder carcinomas another locus on chromosome 13 is the target, as Ishikawa et al. [9] were unable to demonstrate functional loss of RB protein (using Western blotting or immunohistochemistry) in five cases showing LOH at the RB locus. The incidence of altered RB expression reported by Jahnson and Karlsson [10] was 33%. Such variation in results is possibly due to differences in staining techniques and scoring methods.

### DISCUSSION

It has been reported that 50%-70% of patients with bladder cancer experience recurrence after initial successful treatments and about 10-20% of these patients die of the disease despite precise pathologic staging and grading, we are unable to predict clinical outcome in all patients. The retinoblastoma susceptibility gene (RB) a prototype of tumour suppressor genes, has recently been associated with development and/or progression of bladder cancer, as well as sarcoma and small-cell lung cancer [4]. The aim of this study was to investigate the hypothesis that altered patterns of RB expression correlate with prognosis in bladder cancer.

In this study the incidence of altered RB expression was 42.5% Wright et al. [19] found that RB protein was undetectable in 15 (18%) out of 84 bladder cancers. In a series of 48 bladder tumours, Cordon-Cardo et al. [4] scored nine (19%) as negative for RB expression (also assessed immunohistochemically using frozen sections), although their negative group also included tumours showing < 10% positive cells. Both studies may underestimate the proportion of tumours with alterations in the RB gene, as mutations do not necessarily lead to diminished antigenicity. Cairns et al. [3] found a loss of heterozygosity (LOH) at the RB locus in 30% (28 of 94 tumours from informative patients), but it remains possible that at least in some bladder carcinomas another locus on chromosome 13 is the target, as Ishikawa et al. [9] were unable to demonstrate functional loss of RB protein (using Western blotting or immunohistochemistry) in five cases showing LOH at the RB locus. The incidence of altered RB expression reported by Jahnson and Karlsson [10] was 33%. Such variation in results is possibly due to differences in staining techniques and scoring methods.

### Table (1): Correlation of RB protein expression with tumour grade and stage.

<table>
<thead>
<tr>
<th></th>
<th>RB protein</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Altered</td>
<td>Non altered</td>
</tr>
<tr>
<td>Well/moderately differentiated (G1/G2)</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Poorly differentiated G3</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Superficial tumours (Tn, T1)</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>Invasive tumours (T2, T3, T4)</td>
<td>13</td>
<td>4</td>
</tr>
</tbody>
</table>

### Table (2): Ki67 indices and RB status within subgroups of tumour stage, histopathological grade.

<table>
<thead>
<tr>
<th>Ki67 index</th>
<th>n</th>
<th>Range</th>
<th>Mean (± SD)</th>
<th>Altered RB</th>
<th>Non altered RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>23</td>
<td>1-19.6</td>
<td>7 (± 5.5)</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>T2</td>
<td>3</td>
<td>1-16.4</td>
<td>6 (± 4.6)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>T1</td>
<td>20</td>
<td>3.8-19.6</td>
<td>11(± 5.3)</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Invasive</td>
<td>17</td>
<td>1.6-46.9</td>
<td>18.5 (± 11.5)</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>T2</td>
<td>8</td>
<td>13.6-16.7</td>
<td>15 (± 1.2)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>T3</td>
<td>5</td>
<td>2.4-46.9</td>
<td>21 (± 12)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>T4</td>
<td>4</td>
<td>1.6-45.9</td>
<td>22 (± 15.5)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>G1/G2</td>
<td>25</td>
<td>1-20</td>
<td>6.5 (± 4.9)</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>G3</td>
<td>15</td>
<td>1.7-48</td>
<td>19 (± 11.8)</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Altered RB</td>
<td>17</td>
<td>1.2-47.2</td>
<td>20 (± 16.5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non altered RB</td>
<td>23</td>
<td>1-42</td>
<td>10.5 (± 8.4)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Student t test

Superficial V/invasive: p < 0.0001

(G1/G2) V/G3: p < 0.001

Altered RB versus non altered Rb: p < 0.05
The definitions of an altered RB staining differs greatly between studies. Some investigators consider that all nuclei in tumour cells should be negative [19,20] to classify a tumour as having an altered RB expression, while others also include tumours with areas of positively and negatively stained tumour cell nuclei [4,12]. The RB immunostaining has previously been found to be heterogenous as it depends on the cell cycle and therefore, heterogenous staining should not be considered altered [9]. However, standardization of the immunohistochemical procedure also seems warranted for studies of the expression of RB nuclear protein.

In this study, altered RB protein expression was more frequent in muscle invasive, poorly differentiated tumours. Association of RB gene alterations with higher stage have previously been reported [3,4,20].

Baithum and his Colleagues [1] reported that distinctive genetic (low incidence of RB and neurofibromatosis-1-NF-1 abnormality) and kinetic (slower cell turnover profiles) also correlated with "a single file" infiltration pattern and poor survival in muscle invasive transitional cell carcinoma.

In this series the 5-years survival was significantly decreased in patients with altered RB protein compared with survival in patients with normal expression of RB ($p < 0.05$). In a previously reported series, patients with tumours showing altered RB expression (≤ 50% tumour cells positive) had shorter disease-free survival compared with other patients [4]. A similar observation was made by Logothetis et al. [12] who examined muscle invasive tumours from 43 patients using immunohistochemistry.

Similar findings have been reported by Grossman et al. [8] and Cote et al. [5]. The latter found that cases of transitional cell carcinoma of the bladder with undetectable (RBo) and high (RB 2+ i.e. > 50% of tumour cells showing nuclear reactivity) had identical rates of recurrence. These cases had significantly higher recurrence ($p = 0.0001$) and lower survival rates ($p = 0.0002$) compared to cases with moderate (RB 1+ i.e. 1-50% of tumour cells showing nuclear reactivity). Indicating that high levels of RB expression may reflect a dysfunctional (altered) RB pathway.

More recently a characteristic difference was demonstrated between recurrent non invasive and recurrent progressing bladder tumours which is loss of cell regulatory genes including RB in the latter group [16]. However, Jahnson and his associates [11] found that the results of RB immunostaining procedures had no predictive value for tumour response to radiation treatment, local control or cancer specific mortality. In contrast, Mack et al. [13] demonstrated that RB status may predict responsiveness to U.CN-01 (a protein kinase inhibitor) with a potent antineoplastic activity in non-small cell lung carcinoma.

Wada et al. [16] demonstrated that an altered RB function is related to the expected clinical course of urinary bladder cancer, but allelic loss including the gene also occurs in low grade and low stage tumours. They suggested that an altered RB function probably is not necessary for malignant transformation of urothelial cells. The causal direction of the relation between the quantity of deleted DNA and tumour aggressiveness is not clear.

The ki67 index (a measure of tumour growth fraction) varied over a very wide range (1-48.3%), but there were significant differences between mean scores for G1/G2 versus G3 tumours ($p < 0.001$) and for non invasive muscle versus invasive tumours ($p < 0.001$) (Table 2).

Similar findings have been reported in four other studies [2,14,15,19], although the scores obtained by Bush et al. [2] were consistently higher and those by Mellon et al. [14] were lower compared with the present study. Such variation is presumably a consequence of differences in staining techniques and scoring methods.

In this series ki67 indices for tumours with altered expression of RB protein were significantly greater than those with normal RB protein expression ($p < 0.05$). This is consistent with the role for alteration of this gene in the deregulation of cell proliferation in transitional cell bladder carcinoma. Shinohara et al. [17] reported that RB participates in the regulation of the G1/S- phase transition and in P53 mediated apoptosis.

In conclusion altered patterns of RB protein expression may be an important prognostic variable in patients presenting with invasive bladder carcinoma.

These discordant results between different
series might be explained by the differences in antibodies used, techniques of immunostaining, cutoffs for altered immunostaining and counting procedures. To compare between the results from different studies, standardizations of these procedures are necessary. Similarly, interaction between RB protein and other nuclear proteins might explain the discordant results between different studies.

REFERENCES


