

Evaluation of p53, bcl-2 Proteins and DNA Ploidy in Squamous and Transitional Cell Carcinomas of Urinary Bladder

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ABSTRACT

The study covered 33 urinary bladder carcinoma cases, where mutated p53 nuclear protein and bcl-2 gene products were evaluated. Image analysis proved the correlation between genopathologic alterations and DNA ploidy. Immunohistochemistry technique and Feulgen stain methods were applied to paraffin embedded tissue sections. Squamous cell carcinoma (Squ.C.C.) was diagnosed in 19 biopsies and transitional cell carcinoma (T.C.C.) in 14 biopsies. In Squ.C.C group, 10 cases (52.6%) demonstrated nuclear accumulation of mutated p53 protein with significant correlation to the advanced stage and high grade tumors ($p = 0.03$ and 0.0001 , respectively). On the contrary, cytoplasmic expression of bcl-2 was detected in 7 cases (36.8%). Dual expression of both biomarkers was observed in 2 cases. In T.C.C group, a single case (7.1%) was positive for p53 nuclear reactivity and 5 cases (35.7%) demonstrated bcl-2 cytoplasmic expression. In both groups, all p53 positive carcinomas comprised significant correlation to aneuploid histograms of DNA distribution patterns. However, 11 out of 12 bcl-2 positive cases (91.6%) demonstrated proliferative diploid histograms with a wide S-phase. It was concluded that, p53 was significantly expressed in Squ C.C. versus T.C.C. and was associated with advanced stage, high grade and aneuploid DNA. On the other hand, bcl-2 expression displayed insignificant difference in both groups of tumor.

Key Words: *Cancer bladder - p53 gene - bcl-2 - DNA ploidy.*

INTRODUCTION

Bladder tumors, are believed to arise as a consequence of (irreversible lesions in DNA) (initiation) and continued division and proliferation (promotion). Progression towards neoplasia may require cumulative effects of one or more initiating/promoting agents [26]. During the past few years mutations of the tumor suppressor gene p53 have been the most widely studied genetic defect in bladder cancers [9&29]. Tumor suppressor gene (p53) is located on chromosome 17p13 [1] which is the most fre-

quent site for different mutations found in human cancers [19]. Mutant p53 protein can functionally inactivate the wild-type gene product forming mixed complexes that actually has a growth-promoting effect [21]. Mutant p53 has a prolonged half-life and can be demonstrated by immunohistochemical techniques [14]. Proto-oncogene bcl-2 increases cell longevity by allowing the cells to escape apoptosis. The p53 gene alterations and instability block the bcl-2 pathway for apoptotic elimination of DNA damaged cells [23]. In bladder cancer the relationship between p53 and bcl-2 in bladder cancer has not been fully investigated, although both proteins are involved in the regulation of cell cycle and apoptosis. Damage of cell cycle cascade including stabilization of p53 gene, cell cycle arrest, DNA repair and apoptotic pathway allows mutagenic DNA damage and replicating genetic errors [24]. Simultaneous evaluation of these proteins is required for a better understanding of their role in bladder carcinomas. Tumor suppressor gene inactivation and tumor aneuploidy have been studied by few groups. Aneuploid tumors having more frequent allelic loss on chromosome 17p and significant correlation was detected between p53 overexpression and aneuploidy in advanced cases of different carcinomas [8]. The study aimed at evaluating the expression of p53 and bcl-2 proteins in squamous cell carcinoma versus transitional cell carcinomas.

MATERIAL AND METHODS

A total of 33 specimens of bladder biopsies were included in this study. Tissue sections were prepared from 28 radical cystectomies and 5 through cystoscopic procedure. All sections

were fixed in neutral buffered formalin and processed for embedding in paraffin wax. Paraffin sections of 5µm thickness were prepared for hematoxylin and eosin staining, cyto-histochemistry and Feulgen stains. Tumor grading was assessed in all biopsies and postoperative pathologic staging was performed on the cystectomy specimens according to TNM classification [22]. Advanced stage was including P3a, P3b and high grade including grade II and III.

Immunohistochemistry:

Five microns thick sections were mounted on optiplus positive-charged adhesive slides (Biogenix co.). Heat antigen retrieval procedure was carried on by using microwave [20]. Prior to the use of p53 and bcl-2 antibodies, sections were washed in PBS (PH 7.4) and Tris buffer solutions (pH 7.6), respectively. Then sections were incubated with p53 monoclonal antibody BP53-12 clone (1:50 dilution) (Zymed Laboratories Inc, US) and another set of tissues were covered by 1:20 dilution bcl-2 monoclonal antibody (Novo Castra laboratories Ltd, U.K), over night at room temperature. Immunoperoxidase complex development was accomplished with avidin-biotin-peroxidase complex secondary monoclonal universal Histostain kit with DAB chromogen.

Reactivity of p53 displayed nuclear staining and bcl-2 expression was defined by membranous and/or cytoplasmic reaction.

Feulgen stain:

Sections were deparaffinized, rehydrated and refixed in neutral buffered formalin; processed according to Lees et al., [15] and examined by CAS 200 image analysis. Tissue correction to 5 µm thickness was applied to all sections.

The histograms were classified according to Lees et al., [15] into: Diploid histogram (A area contained less than 75% of examined cells). Proliferative diploid histogram (B area < 40%). Aneuploid peaks (B area > 50%. D and F areas > 10%) and multiploid based on multiple distinct peaks > 10% present in different areas of E area included > 10% of examined cell population.

Data analysis:

Tumor histologic type, grade, stage and DNA distribution patterns, were correlated to

the expression of mutated p53 and bcl-2 proteins. Coding of data was carried out manually and statistical analysis was conducted through EPI-EMFO on IBM compatible computer. Tests of significance were; *t*-test, Chi square and Anova analysis of variance. The level of significance was 5% ($p < 0.05$).

RESULTS

A total of 33 cases of bladder carcinoma were examined. Squamous (Squ.C.C.) was diagnosed in 19 cystectomy specimens. Transitional cell carcinoma (T.C.C.) was present in 14 cases, of which 6 cases demonstrated the papillary variant (Figs. 1 & 2).

In Squ.C.C. group: Superficial infiltration (stage P2) was seen in one case, while advanced stages (P3a and P3b) were present in 18 cases. Low grade cellular differentiation (GI) was detected in 5 cases and high grade (GII & GIII) in 14 cases.

In T.C.C. group, superficial invasion was seen in 5 cases and the 9 cystectomy specimens exhibited advanced stages. Low grade was present in 4 cases and high grade in 10.

Oncopathology:

The relations among both oncoproteins biomarkers (p53 and bcl-2) reaction to the tumor type, stage and DNA ploidy are presented in (Table 1).

P53 results:

Heterogeneous nuclear reactivity was noticed in 10 cases out of the 19 Squ.C.Cs (52.6%) (Fig. 1c). These constituted 55.5% of the 18 advanced stage cases. Nine out of 13 high grade cases (69.2%) exhibited positive reaction.

Out of 14 T.C.C. cases, a single case was positive for mutated p53 (7.1%). It was associated with advanced stage of infiltration and high grade of cellular differentiation.

bcl-2 oncoprotein results:

Cytoplasmic and/or membranous expression was detected in 5 out of 19 Squ.C.Cs (26.3%). Positive reaction was noticed in 2 out of 3 superficial tumors (66.6%) and 3 out of 11 advanced stage cases (27.27%). Low grade tu-

mors (2 cases) exhibited positive reaction in a single case (50%) and in 4 out of 8 high grade cases (50%).

Among T.C.Cs 5 cases (35.7%) demonstrated bcl-2 expression (Fig. 2). Positive reactivity was noticed in the 2 cases of superficial infiltration and in 3 out of 8 advanced stage cases (37.5%). One out of 3 low grade (33.3%) and 4 out of 11 high grade cases (36.3%) displayed positive reaction.

Dual expression of p53 and bcl-2 results:

Positive reactivity of both markers was detected in 2 of the Squ.C.C. cases (10.5%). They were associated with advanced stage and high tumor grade.

Statistical analysis revealed significant increased incidence of mutated p53 expression in Squ.C.C. versus T.C.C. ($p = 0.0006$). P53 expression was significantly correlated to ad-

vanced stage ($p = 0.03$) and high grade of both tumor variants ($p = 0.0001$). Bcl-2 expression showed no significant correlation to tumor types ($p = 0.72$), stage ($p = 0.23$) or grade ($p = 0.09$) (Fig. 3).

Evaluation of DNA ploidy is presented in (Fig. 4). Evidence of DNA damage was noticed with p53 positive cases irrespective to its histologic type. All positive cases displayed aneuploid and multiploid patterns and significantly correlated to Squ C.C. variant ($p = 0.0001$). However, increased proliferative activity and DNA synthesis were reflected by wide S-phase in 11 out of 12 bcl-2 positive carcinomas (78.5%) (Table 2). Aneuploid peaks were detected in the D area (contained more than 10% of studied cell population). Multiploid histogram showed multiple stimline peaks in different areas (D and F), which contained 38.04% and 35.87%, respectively of the studied cell population.

Table (1): Correlation of biomarkers expression to histopathology, staging and grading of cancer bladder.

Biomarkers	Squ.C.C (19 cases)				T.C.C (14 cases)			
	Stage		Grade		Stage		Grade	
	Superficial (1)	Advanced (18)	Low (5)	High (14)	Superficial (4)	Advanced (6)	Low (4)	High (10)
P53 (11 cases)	1	9	1	9	0	1	0	1
bcl-2 (12 cases)	1	6	4	3	4	1	1	4
Dual reactivity (2 cases)	0	2	0	2	-	-	-	-

Table (2): DNA distribution pattern in biomarkers positive cases.

DNA ploidy	Postiive p53 (11 cases)	Positive bcl-2 (12 cases)	Dual reaction (2 cases)
Prolif. diploid	-	11	-
Aneuploid	11	1	-
Multiploid	-	-	2

Prolif. Diploid: Proliferative Diploid.

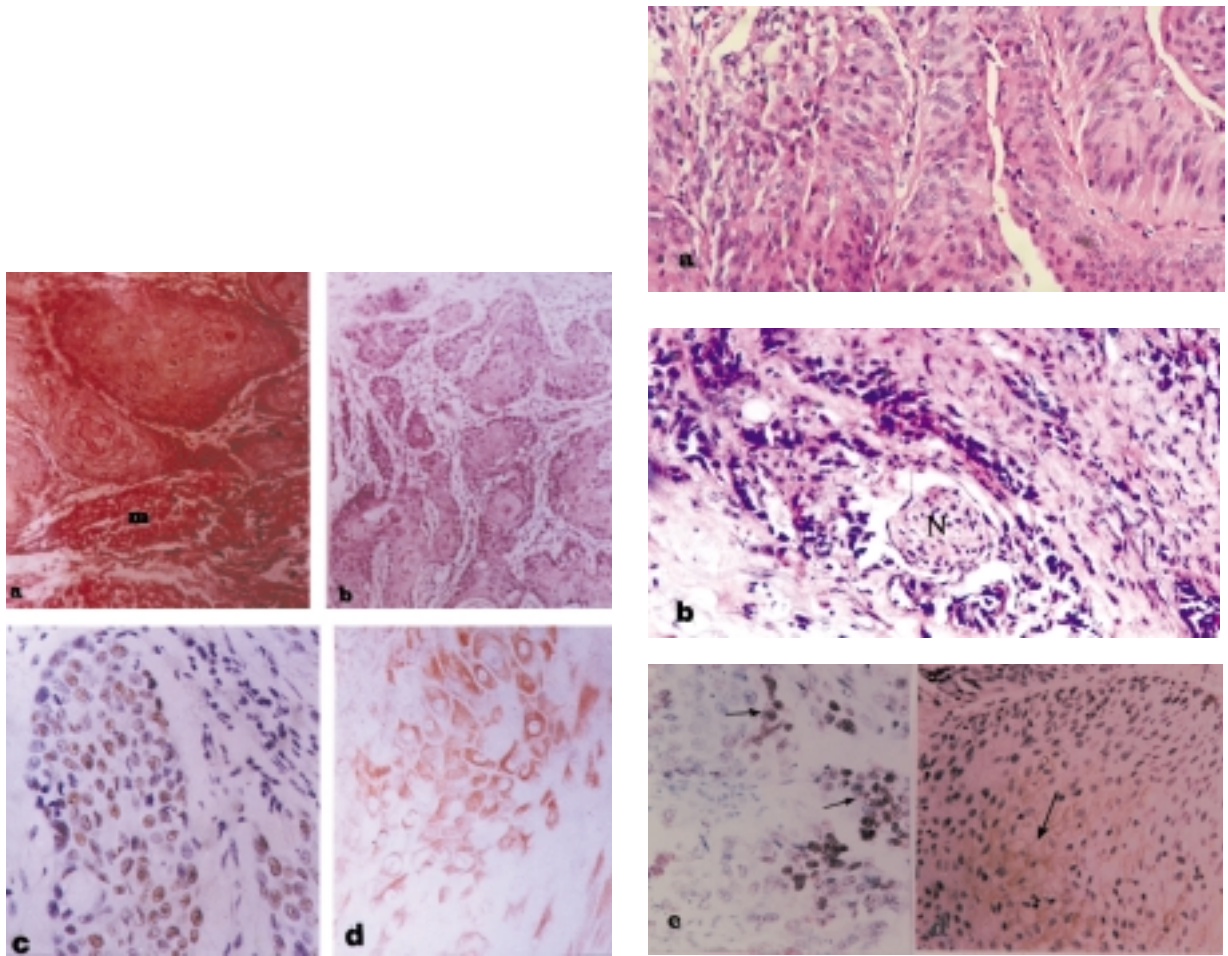


Fig. (1): a&b- Squamous cell carcinoma high grade with muscle invasion (H & E X 400). c- Frequent strong heterogeneous p53 nuclear reactivity. d- Evident cytoplasmic expression of bcl-2 in low grade case. (Immunoperoxidase, DAB X 400 chromogen).

Fig. (2): a- papillary transitional cell carcinoma high grade. b- Transitional cell carcinoma showed deep muscle and perinural invasion (N). c- Strong p53 nuclear reactivity (arrows). d- Cytoplasmic and membranous expression of bcl-2 product in well differentiated transitional cell carcinoma. (Immunoperoxidase, DAB, X 400).

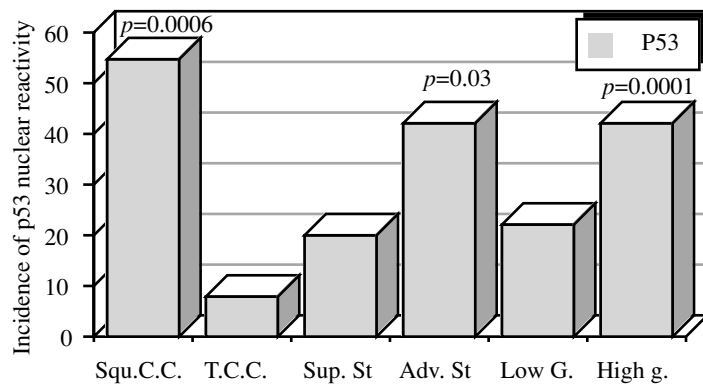


Fig. (3): Relation of p53 reactivity to type stage and grade.

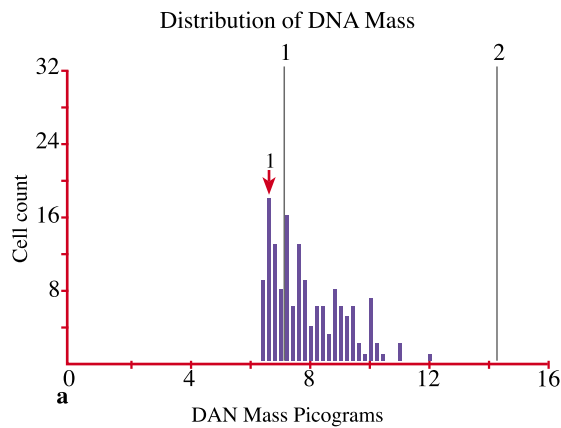


Fig. (4,A)

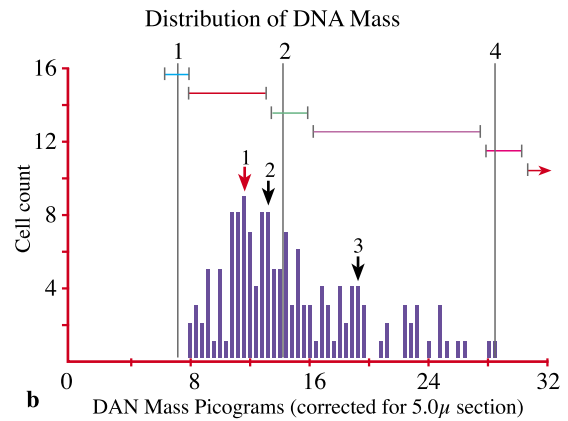


Fig. (4,B)

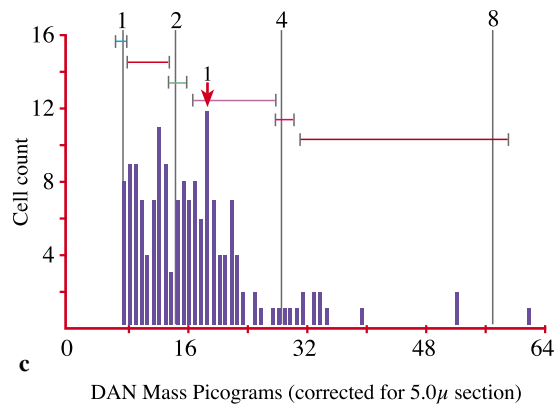


Fig. (4,C)

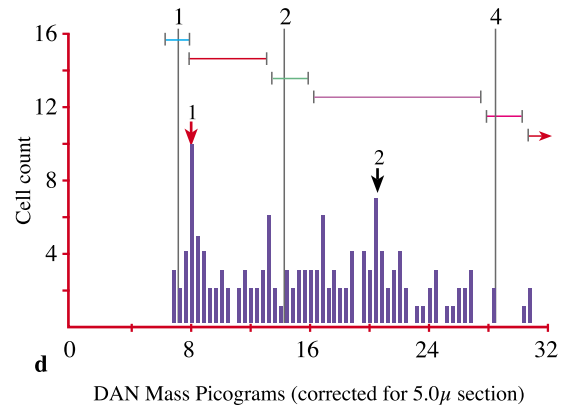


Fig. (4,D)

Fig. (4): Histograms of DNA distribution patterns analysed by CAS 200 image analyser showing:

- a- Proliferative diploid histogram in bcl-2 positive bladder carcinomas.
- a- Aneuploid histogram of squamous cell carcinoma with evident p53 nuclear reactivity, the peaks were detected in B area (47.59%) and D area (23.49%) (arrows).
- b- Multiploid histogram of squamous cell carcinoma low grade with evident bcl-2 and weak p53, showed multiple peaks in C area (23.42%), D area (23.42%) and F area (29.75%) (arrows).
- d- Multiploid histogram in transitional cell carcinoma grade III, showed multiple distinct peaks in D area (44.59%) and F area (21.02%) as well as E area (10.19%).

DISCUSSION

Bladder neoplasms show a wide variation in the natural course and prognosis. Determining specific biochemical markers for the changes resulting from the process of carcinogenesis offers an attractive approach to identify individuals at risk for bladder cancer. The genotypic and phenotypic characteristics of a tumor represent the end product of a complex evolutionary process driven by genetic instability and epigenetic factors [1]. During the last few years, mutation of the tumor suppressor gene p53 has been the most widely studied genetic defect in bladder cancer [10]. Biomarkers may assist in delineating reactive inflammatory atypia from premalignant dysplasia and may serve to target

early chemopreventive strategies before irreversible genetic alterations associated with carcinogenesis [1]. In this study, we investigated the immunoreactivity of both p53 and bcl-2. The proto-oncogene and biomarkers evaluation in transitional cell carcinoma have been widely investigated [16,27]. On the contrary, p53 suppressor gene alterations in squamous cell carcinoma have not received much attention. Immunoreactivity for the mutated p53 protein has been reported in 54% of bladder carcinomas of transitional cell type in the study of Wright et al. [30]. In the present study, significantly increased p53 nuclear reactivity was noticed in Squ.C.C. versus T.C.C.; in parallel with tendency of mutated p53 to accumulate frequently when the tumor begins to invade muscle [18,29]. In concordance

with the present results, significant correlation was noticed with advanced stage and high grade tumors. P53 is considered as a prognostic marker, that might reflect aggressive malignant potential, invasiveness and poor prognosis [6].

Apoptosis inhibitor bcl-2 oncoprotein product carries less risk of invasion potentiality and its relationship to tumor grade is controversial [29]. In the series of Bilim et al. [3] and Glick et al. [13], variable degrees of bcl-2 cytoplasmic reactivity were noticed. Parallel to these observations, the current study comprised non significant correlation to stage or grade. Although the biological explanation for difference between transitional and squamous carcinomas is unclear, possibly bcl-2 protein might play a role in different types of neoplasms [17]. It was found that bcl-2 positivity had a favorable prognostic impact in some tumors and unfavorable in others [4,5,17]. Moreover, the difference noted in the present study between transitional and squamous tumors in their expression of both markers might represent a difference in the underlying genetic error.

In the present study, squamous cell carcinoma showed inverse relation of bcl-2 expression to p53 nuclear reactivity; the highly positive bcl-2 displayed weak nuclear reactivity to p53. A reciprocal relationship between bcl-2 and p53 was reported [7&29]. Reciprocal staining was also detected in prostatic adenocarcinoma [2] and others [7&28]. Parallel to our observation, co-expression of both bcl-2 and p53 was found to occur in some bladder tumors [11&12]. Current squamous cell carcinomas were associated with advanced stage and grades.

In a retrospective study on the impact of DNA in predicting progression of the disease, Wrinkler and coworkers [30] demonstrated insignificant correlation to tumor grade but DNA ploidy is in concordance with stage. On the contrary, in the study of Shaaban and associates [25] a good correlation between tumor grade rather stage with DNA ploidy was observed. The relationship between tumor suppressor gene inactivation and tumor ploidy has been studied by few investigators [8].

The present study established significant correlation between p53 mutation and tumor aneuploidy or multiploidy, whereas, bcl-2 expression was correlated to proliferative diploid pattern of DNA distribution. Heterogeneity and

gene instability which were demonstrated by heterogeneous nuclear reactivity could be attributed to the underlying mechanism of multiploidy. Inactivated p53 gene is associated with DNA aneuploidy in different carcinomas [18&19].

In conclusion, squamous cell carcinomas displayed significant higher p53 positivity versus T.C.C. and was associated with advanced stage, high grade and aneuploid pattern of DNA. P53 could be considered as a prognostic tumor marker. Different behavior and underlying genetic error might be involved in the tumorigenesis.

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