Alterations of p53 and Rb Pathways Are Associated with High Proliferation in Bladder Urothelial Carcinomas

ANNA C. GOUSSIA¹, ALEXANDRA PAPOUDOU-BAI¹, ANTONIA CHARCHANTI², PANAGIOTIS KITSOULIS²,
PANAGIOTIS KANAVAROS², JOHN KALEF-EZRA³, DIMITRIOS STEFANOU¹ and NIKI J. AGNANTIS¹

*Departments of ¹Pathology, ²Anatomy-Histology-Embryology and ³Medical Physics Medicine,
Medical Faculty, School of Health Sciences, University of Ioannina, Ioannina, Greece*

Abstract. *Background/Aim:* Since most cancers are associated with alterations of the p53 and Rb pathways, the expression of p53, p21, Rb, p16, p27, cyclin D1, cyclin A, cyclin B1 and Ki67 proteins were analyzed in bladder urothelial carcinomas (BUC). *Materials and Methods:* One hundred twenty-two cases of BUC were studied by immunohistochemistry. *Results:* The pathways p53/p21 and Rb/p16/cyclin D1 exhibited alterations in 81/115 and 63/84 cases, respectively. Alterations of the p53/p21 and Rb/p16/cyclin D1 pathways were positively correlated with high cyclin A expression. High expression of p53, Ki67, cyclin A and cyclin B1 was inversely correlated with the papillary morphology of the tumor and positively with tumor grade and T-stage. *Conclusion:* The results showed that a) alterations of the p53 and Rb pathways are associated with high proliferation of tumor cells in BUC and b) high expression of cell-cycle proteins is associated with adverse histopathological parameters of these tumors.

Cell-cycle progression is regulated by a complex molecular network involving cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (CDKIs) (1). The p53 and Rb growth suppressor pathways play important roles in the regulation of the cell cycle (1). P53-dependent G₁-S and G₂-M cell-cycle arrest is mediated, at least in part, through p53-mediated induction of the CDKI p21 and by repression of the promoters of cyclin B1 and CDK1, respectively (1). The Rb pathway is involved in the inhibition of transcription of genes necessary for the G₁-S transition (1). Hypophosphorylated Rb protein (pRb) binds and inactivates transcription factors,

notably the E2F1, important for the G₁-S transition. As the cycle proceeds, pRb becomes hyperphosphorylated and loses its ability to bind to E2F1. The phosphorylation of pRb is achieved by the cyclin D-CDK4/6 complexes and inhibited by the CDKI p16 (1).

Abnormalities of cell-cycle components are common in bladder urothelial carcinomas (BUC) and may be relevant with respect to the pathogenetic and clinical behaviour of these tumors (2-37). For example, p53 mutations and/or protein overexpression were significantly related with tumor grade, stage and the presence of lymph node metastases (2, 4). p21 protein expression was an independent predictor of tumor progression (11). Moreover, patients with low p21 expression had shorter recurrence-free survival and overall survival rates (30). Interestingly, patients with p53+/p21- tumors had a higher rate of recurrence and worse survival than those with p53+/p21+ tumors (7). Altered pRb protein expression occurs in all grades and stages of BUC but was more commonly noticed in higher grade and muscle invasive tumors (3). The immunohistochemical overexpression of pRb protein has been associated with increased rate of progression and decreased survival (6). Abnormal expression of the p16 and cyclin D1 proteins has been involved in urothelial tumorigenesis and tumor recurrence (12, 15, 18). Importantly, the results of a meta-analysis showed a link between downregulated p16 expression and poor prognosis in patients with BUC in terms of recurrence-free survival (RFS), overall survival (OS), progression-free survival (PFS), and some clinicopathological parameters including clinical staging, pathological grade and lymph node metastasis (31). Reduced expression of p27 protein has been associated with aggressive and highly proliferating BUC, as well as with unfavorable prognosis (9, 13).

Interestingly, concomitant alterations of various cell-cycle regulators have been associated with tumor recurrence and aggressive behavior of BUC (5, 19, 24, 37), but the impact of such concurrent alterations on tumor cell proliferation, to the best of our knowledge, has not been analyzed so far. Thus, the expression of p53, p21, pRb, p16, p27, cyclin D1,

Correspondence to: Anna Goussia, MD, Ph.D., Department of Pathology, Medical Faculty, School of Health Sciences, University of Ioannina, University Campus, 45110 Ioannina, Greece. Tel: +30 2651099413, e-mail: agoussia@uoi.gr

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cyclin A, cyclin B1 and Ki67 proteins in 122 cases of BUC were investigated in order: a) to search for immunohistochemical alterations of these cell cycle regulatory proteins, b) to determine the proliferation status (expression of cyclin A, cyclin B1, Ki67), c) to analyze the impact of concurrent immunohistochemical alterations of p53 and Rb growth suppressor pathways on tumor cell proliferation and d) to correlate the findings with histopathological parameters.

Materials and Methods

Tissue samples. A total of one hundred twenty-two (122) cases of BUC was selected from the files of the Department of Pathology, University Hospital of Ioannina on the basis that fixed tissue was available for performing multiparametric immunohistochemical analysis. Cases were assigned into two grade categories (low and high) and in each case the tumor configuration (papillary/nonpapillary) and the pathological stage (pT) were recorded.

Immunohistochemistry. Immunohistochemical analyses were performed on paraffin-embedded tissue sections by the labelled streptavidin avidin biotin method (LSAB kit, Dako SA, Glostrup, Denmark). Pretreatment of the sections with 10 mM sodium citrate buffer (pH 6.0) in a microwave oven was performed. Monoclonal antibodies directed against p53 protein (DO-7; Dako SA; dilution 1:50), p21 (NCL-WAF-1; Novocastra, Newcastle upon Tyne, UK, dilution 1:20), pRb (Ab-5; Oncogene, CA, USA; dilution 1:50), p16 (F-12; Santa Cruz Biotechnology, Texas, USA; dilution 1:50); p27 (IB4; Novocastra; dilution 1:50), cyclin D1 (DCS-6; Novocastra; dilution 1:50), cyclin A (6E6; Novocastra; dilution 1:50), cyclin B1 (7A9; Novocastra; dilution 1:10) and Ki67 (MIB-1; Dako SA; dilution 1:50) were applied. Positive control slides were included and consisted of thymus and non-Hodgkin lymphomas from our previous studies (38, 39). Negative controls consisted in the same immunohistochemical method with omission of the primary antibody. A continuous score system was adopted and the number of immunopositive cells was divided by the total number of the counted cells and the expression was defined as the percentage of positive cells in the total number of the counted cells. p53, p21, Rb, p16, cyclin D1 and p27 aberrant expression patterns were determined on the basis of previous published criteria (20, 22, 24).

Statistical analysis. The program SPSS for Windows Release 10 was used and the results were considered as statistically significant when $p < 0.05$.

Results

Expression of p53, p21, pRb, p16, p27, cyclin D1, cyclin A, cyclin B1 and Ki67 proteins and correlations with histopathological parameters. The non-neoplastic urothelium expressed Ki67, cyclin A, cyclin B1, pRb, p16, p21 and p27 proteins but was negative for p53 and cyclin D1.

High expression (>10% of neoplastic cells) levels of the proteins p53, p21, pRb, p16, p27, cyclin D1, cyclin A, cyclin

B1 and Ki67 were found in 79/122 (64.75%), 20/121 (16.53%), 51/115 (44.35%), 74/95 (77.9%), 80/88 (90.9 %), 39/120 (32.5%), 38/113 (33.63%), 6/97 (6.18%), 57/101 (56.43%) of the cases, respectively.

Using the Spearman's correlation coefficient test, significant positive correlations were found between p53/p21 ($p=0.001$), p53/cyclin A ($p<0.001$), p53/Ki67 ($p=0.005$), p21/cyclin D1 ($p<0.001$), p21/cyclin A ($p=0.006$), pRb/cyclin A ($p=0.003$), pRb/cyclin B1 ($p=0.031$) and cyclin D1/cyclin A ($p=0.048$).

High expression (>10% of neoplastic cells) levels of p53, cyclin A, cyclin B1 and Ki67 proteins were inversely correlated with the papillary morphology of the tumor ($p=0.026$, $p<0.0001$, $p<0.0001$, and $p=0.001$, respectively) and positively with tumor grade ($p=0.014$, $p<0.0001$, $p=0.002$, and $p=0.027$, respectively) and pT-stage ($p=0.014$, $p<0.0001$, $p=0.004$, and $p=0.025$, respectively).

Alterations of the p53/p21 and Rb/p16/cyclin D1 growth suppressor pathways. The normal and aberrant p53/p21 and Rb/p16/cyclin D1 patterns are shown in Tables I and II. Totally, aberrant p53/p21 patterns were observed in 81/115 (70.43%) cases and aberrant Rb/p16/cyclin D1 patterns were observed in 63/84 (75%) cases. Concomitant alterations of p53/p21-Rb/p16/cyclin D1 pathways were found in 40/77 (51.95%) of cases.

Correlations of the combined alterations of the p53 and Rb growth suppressor pathways with proliferation profile and histopathological parameters. The combined expression patterns of p53/p21 and Rb/p16/cyclin D1 were analyzed with respect to the expression of cyclin A, cyclin B1 and Ki67 proteins. Using analysis of variance, the aberrant p53/p21 phenotypes were positively correlated with cyclin A expression ($p=0.002$) and the aberrant Rb/p16/cyclin D1 phenotypes were positively correlated with cyclin A expression ($p=0.04$).

Discussion

In the present study, high expression (>10% of neoplastic cells) levels of p53, cyclin A, cyclin B1 and Ki67 proteins were inversely correlated with the papillary morphology of the tumor and positively correlated with tumor grade and pT-stage. These results are in keeping with previous studies (25, 32, 37) and show that high expression of cell cycle proteins such as p53, cyclin A, cyclin B1 and Ki67 is associated with adverse histopathological parameters.

In our study, immunohistochemical alterations of the p53/p21 and Rb/p16/cyclin D1 pathways were found in 81/115 (70.43%) and 63/84 (75%) of the cases, respectively. These findings are in keeping with previous immunohistochemical data (10, 24) and indicate that a large number

Table I. *p53/p21 expression patterns.*

Patterns	Number of cases	
p53+/p21+	16/115 (13.91%)	Aberrant
p53+/p21-	61/115 (53.04%)	Aberrant
p53-/p21-	4/115 (3.48%)	Aberrant
p53-/p21+	34/115 (29.56%)	Normal
Total cases	81/115 (70.43%)	Aberrant/total

Cut-off of positivity: the expression of the proteins in at least 10% of neoplastic cells was considered positive expression.

Table II. *Rb/p16/cyclin D1 expression patterns.*

Patterns	Number of cases	
Rb+/p16+/cyclin D1-	21/84 (25%)	Normal
Rb+/p16-/cyclin D1-	3/84 (3.57%)	Aberrant
Rb-/p16+/cyclin D1-	23/84 (27.39%)	Aberrant
Rb-/p16-/cyclin D1-	9/84 (10.71%)	Aberrant
Rb+/p16+/cyclin D1+	8/84 (9.52%)	Aberrant
Rb+/p16-/cyclin D1+	6/84 (7.14%)	Aberrant
Rb-/p16+/cyclin D1+	13/84 (15.48%)	Aberrant
Rb-/p16-/cyclin D1+	1/84 (1.19%)	Aberrant
Total	63/84 (75%)	Aberrant/total

Cut-off of positivity: the expression of the proteins in at least 10% of neoplastic cells was considered positive expression.

of BUC display alterations of at least one of the growth suppressor pathways. Furthermore, concomitant alterations of p53/p21-Rb/p16/cyclin D1 pathways in 40/77 (51.95%) of the cases were observed. These findings indicate that concurrent alterations of the p53/p21 and Rb/p16/cyclin D1 pathways are frequent in BUC and may play important roles in their biological behavior. Indeed, alterations in both p53 and Rb may act cooperatively to promote bladder cancer progression (5, 6, 19). The novel finding of the present study is that the alterations in the p53/p21 and Rb/p16/cyclin D1 expression status were significantly associated with high expression levels of cyclin A. The above data suggest that the impairment of p53 and Rb pathways in BUC results in enhanced tumor cell proliferation.

In conclusion, our results show that a) alterations of the p53 and Rb pathways are common and associated with high proliferation of tumor cells in BUC and b) high expression of cell cycle proteins such as p53, cyclin A, cyclin B1 and Ki67 is associated with adverse histopathological parameters of these tumors.

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