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Bladder Cancer

Molecular and Immunohistochemical Analysis of the Prognostic Value of Cell-Cycle Regulators in Urothelial Neoplasms of the Bladder

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Abstract

Objective: To evaluate the prognostic and predictive value of molecular and immunohistochemical markers related to cell-cycle control in terms of recurrence, progression, and survival in urothelial neoplasms of the bladder (UNB).
Patients and Methods: Clinical and pathological findings of 84 patients with UNB were assessed. Homozygous deletion (HD) and promoter methylation of $p14^{ARF}$, $p15^{INK4B}$, $p16^{INK4A}$, loss of heterozygosity of the locus 9p21, *p53* mutations, and immunohistochemical expression of p53, p16, p14, p21, p27, pRb, Ki67, MDM2, and cyclin D1 proteins were evaluated in relation to overall survival (OS), recurrence-free survival (RFS), and progression-free survival (PFS).
Results: In the univariate analysis, RFS was shorter in cases with $p14^{ARF}$ ($p = 0.006$), $p15^{INK4B}$ ($p = 0.003$), $p16^{INK4A}$ ($p = 0.03$) HD, low p14 immunoreactivity index (IRI) ($p = 0.01$) and high Ki67 IRI ($p = 0.04$); HD of the 9p21 locus genes and p14 IRI remained as independent prognostic factors for early UNB recurrence ($p = 0.006$) whereas tumour stage ($p = 0.00001$) and cyclin D1 IRI ($p = 0.049$) were related to worse PFS in the multivariate analysis. In the univariate analysis, IRI for Ki67 ($p = 0.002$), cyclin D1 ($p = 0.06$), p53 ($p = 0.00008$), p16 ($p = 0.02$), p27 ($p = 0.0005$) MDM2 ($p = 0.01$) and p53 mutations ($p = 0.03$) were related to poor OS, and only the Ki67 IRI retained their independent value in the multivariate analysis.
Conclusion: : 9p21 HD and p14 IRI constitute independent predictive factors for UNB recurrence and cyclin D1 IRI and tumour stage for progression. In addition, Ki67 IRI and tumour stage are independent prognostic factors for overall survival in UNB.

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1. Introduction

Molecular alterations of genes implicated in cell cycles are important molecular events in carcinogenesis of urothelial neoplasm of the bladder (UNB). The prognostic significance of alterations of the *9p21* chromosomal region, where *p15*^{INK4B}, *p16*^{INK4A}, and *p14*^{ARF} genes are located, as well as alteration of *p21*^{Cip}, *p27*^{Kip1}, *p53*, *MDM2*, and *CCND1* genes and proteins have been intensively studied during the last decade. Numerous studies show them as a prognostic markers of UNB clinical outcomes, but very often results of studies are dissimilar and the comparative prognostic value of these proteins and genes are not clear.

Alterations of *9p21* locus, especially *p16*$^{INK4A/ARF}$ gene, are very promising prognosticators of clinical outcomes because they affect both the p53 and pRb cell-cycle regulatory pathways [1]. The significance of this locus for tumourigenesis was shown with animal models of cancer [2].

The aim of the present study was to evaluate the significance of alterations in 10 cell-cycle regulators to predict recurrence, progression, and overall survival (OS) in a series of patients with UNB.

2. Materials and methods

We analyzed material from 84 patients with UNB who had been treated in the Fundación Instituto Valenciano de Oncología (I.V.O.), Valencia, Spain. The classification of tumour stage and grade was defined according to International Society of Urological Pathology and World Health Organization criteria [3]. No special patient selection was performed, but many Ta tumours were too small to obtain adequate samples and were not included in the study. For the molecular study, fresh-frozen material from the tumour and from bladder urothelium of normal appearance were used. The frozen sections were studied with hematoxylin and eosin. The immunohistochemical (IHC) study was carried out on formalin-fixed, paraffin-embedded tumoural tissues from the same samples.

Tumour progression was defined as: (a) progression from superficial UNB when it becomes a muscle-infiltrating tumour during follow-up; (b) progression from muscle-infiltration UNB when after treatment (cystectomy) the disease has spread to lymph nodes, metastasis, or has had local recurrence.

2.1. Immunohistochemical study

An IHC study for p53, MDM2, pRb, Cyclin D1, Ki67, p14^{ARF}, p16^{INK4A}, p21$^{WAF1/Cip1}$, and p27^{Kip1} proteins was performed with the standard avidin-biotin peroxidase complex (ABC) method with the LSAB kit (DAKO, Denmark). The antibodies used are listed in Table 1. As controls, known positive tissue sections were used, and for negative controls, exposure to the primary antibody was omitted. Colour was developed with 3′,3′-aminobenzidine and counter-stained with Mayer's hematoxylin.

Table 1 – Antibodies used in the study

Antigen	Clone	Source	Retrieval	Dilution
p53	DO7	DAKO	yes	1/50
Ki67	MIB1	DAKO	yes	1/50
p14^{ARF}	AB-4	Neomarkers	yes	1/100
p16^{INK4A}	F-12	Santa Cruz	yes	1/500
p21^{WAF1}	F-5	Santa Cruz	yes	1/50
p27^{KIP1}	SX53G8	Novocastra	yes	1/50
pRb	1F8	Neomarkers	yes	1/25
MDM2	1B-10	Novocastra	yes	1/50
Cyclin D1	P2D11F11	Novocastra	yes	1/20

To evaluate the extent of immunostaining we used the immunoreactivity index (IRI), which was calculated as the percentage of stained cells by counting 1000 cells per slide.

2.2. DNA extraction

DNA was extracted from 0.10–0.15 g of fresh frozen tissue. Lysis solution that contained proteinase K (Life Technologies) in 100 μg/ml concentration was used with subsequent incubation at 37 °C for 16 h. Standard phenol/chloroform protocol, followed by precipitation with ethanol, was used for DNA purification [4]. DNA was quantified by spectrophotometry at 260 nm and quality was checked by measuring the ratio between the 260 nm and 280 nm values.

2.3. Homozygous deletions of p15INK4B, p16INK4A and p14ARF genes

Differential polymerase chain reaction (dPCR) was used to detect homozygous deletions (HD) of 9p21 locus genes. PCR parameters and primer sequences were designed as previously described by our group [5,6]. The PCR products were visualized and photographed in ultraviolet light after electrophoresis in 1.5% agarose (Fig. 1). The gel image was then analyzed by 1D Image Analysis Software (Eastman Kodak). The intensities of the target band and the control band (β-globin) were compared. The presence of HD was determined by decreasing the ratio of target band intensity to β-globin band intensity compared with the normal control samples analysed in the same gel.

2.4. Methylation-specific PCR

The methylation status of the 5′CpG islands in genes promoter regions was determined by following the procedure described by Herman et al. [7]. The primer used and the PCR parameters have been described by our group in previous studies [5,6]. PCR products were analyzed in 2% ethidium-bromide stained gel. We confirmed the presence of hypermethylation by identifying the PCR bands in the samples amplified with the methylated primers. The presence of bands in cases that were amplified with unmethylated primer was used to confirm the quality of the modified DNA.