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Immunoreactivity of bcl-2, p53 and EGFr is associated with tumor stage, grade and cell proliferation in superficial bladder cancer

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Abstract Immunoreactivity of bcl-2, p53, the epidermal growth factor (EGFr) and Ki-67 (MIB-1) proteins was assessed by immunohistochemistry in 185 patients with superficial bladder cancer (SBC) in order to evaluate their usefulness as indicators of tumor progression. Forty-one percent of the tumors were bcl-2 positive, 36% of them were positive for p53 (over 20% of nuclei), while 41% were positive for EGFr, and 30% of the tumors were MIB-1 positive (proliferation index > 15%). Immunoreactivity of all analyzed proteins was highly significantly related to tumor grade and stage. Tumors which were bcl-2, p53 or EGFr positive were also rapidly proliferative (MIB-1 score >15%). The obtained results suggest that all analyzed proteins may have prognostic significance in SBC. The prognostic value of the abnormal immunolabeling of the analyzed proteins will be established after an adequate follow-up period of this same cohort of patients.

Key words Superficial bladder cancer · bcl-2 · p53 · EGFr · MIB-1

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Introduction

Previous studies indicate that during 3 years 10% of superficial bladder cancers (SBC) progress to an invasive disease [17]. Various pathological and clinical parameters have been related to the likelihood of progressive disease. High tumor grade, lamina propria invasion, atypia elsewhere in the bladder epithelium, tumor multiplicity and large tumors are associated with adverse prognosis [12]. The role of subjective histological grading as a prognostic factor is, however, a matter of controversy due to its low interobserver reproducibility [27]. Numerous studies have been performed in order to find better prognostic indicators for patients at high risk of developing progressive disease. The growth rate of neoplasms depends on the proliferation and death rates of cancer cells, which in part may represent programmed cell death or apoptosis [1, 22, 31]. Previous analyses show that proliferation indices and mechanisms related to regulation of cell proliferation have a prognostic potential in SBC [21]. A number of oncogenes and suppressor genes may also influence bladder cancer behavior [8, 9, 19, 20, 26, 44]. The epidermal growth factor receptor (EGFr) overexpression correlates with tumor progression in SBC [26]. In the current study, which is part of a prospective randomized series, the immunoreactivity of p53, bcl-2 and EGFr is compared with established prognostic factors (stage, grade) and the immunoreactivity of proliferation marker MIB-1 (Ki-67) in order to assess their potential usefulness as prognostic indicators in SBC.

Materials and methods

Patients

From December 1991 to March 1994 in 23 Finnish hospitals (the Finnbladder III Group) altogether 273 patients with newly diagnosed superficial transitiocellular carcinoma of the urinary bladder were randomized into three different groups of treatment. In this Finnbladder III trial, one group was treated by transurethral resection (TUR) alone, the second group of patients had an instillation of 50 million IU interferon alpha-2b (IntronaR, Schering-Plough) for 2 h after TUR and the third group received instillation of 100 mg epirubicin (FarmorubicinR, Pharmacia) for 2 h after TUR. The primary diagnostics and staging were carried out according to the UICC 1978 classification [40]. The initial staging was based on urethrocystoscopy, transurethral ultrasound of the bladder, cytological examination of voided urine and excretory pyelography. Pathological staging and grading were performed according to the WHO classification [25]. Slides of the tissue blocks from each participating hospital were sent to the referee pathologist (M.H.) to obtain a uniform diagnosis of T category and grade. The total number of eligible patients was reduced to 207 due to protocol violation, change in T category by the referee pathologist and insufficient sample material. All analyzed parameters of the current study were evaluable in 185 cases.

Histological methods

The histological samples were either preoperative bioptic or peroperative TUR specimens. They were fixed in buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological examination. The samples were histologically graded according to the WHO [25] in a blinded manner, i.e., with no knowledge of the clinical data.

Immunohistochemistry

Ki-67 nuclear antigen (MIB-1)

From routine processed representative paraffin blocks 5-μm sections were cut and placed on poly-*l*-lysine coated slides. Overnight drying of the sections at 37 °C was followed by dewaxing and hydration. The sections were dewaxed in xylene and rehydrated in a graded series of ethanol to water. Citrate buffer (pH 6.0) was used for antigen retrieval in a microwave processor. The sections were treated twice for 7 min at 850 W power in a household microwave oven, after which the sections were allowed to cool in the buffer for 30 min. For the immunostaining of Ki-67 antigen, the monoclonal antibody MIB-1 (IgG1, Immunotech S.A. Marseille, France) was used at a 1:40 concentration. The sections were incubated at +4 °C overnight, and the primary antibody was demonstrated with a streptavidin-biotin technique (Zymed Laboratories Inc., California, USA). Diaminobenzidine was used as the final chromogen. The counterstaining was performed using 0.4% ethyl green in acetate buffer for 15 min.

Quantitation of immunohistochemistry

The stainings were evaluated by one observer (T.L.) using a computer-assisted image analysis system (CAS-200 Software, Beckton Dickinson, USA). The microscope-based system was equipped with two cameras that convert the image of immunopositive areas (brown) and immunonegative areas (green) in nuclei to computer processing. The proliferation index was the percentage ratio of brown and green images. In this study, the index defines the percentage area of immunopositivity in nuclei (area-related proliferation index).

The microscopic fields (x400, $n = 20$) had to be representative of the proliferative activity of the tumor tissue. Only neoplastic cells were included in the analysis. Necrotic and technically poor (damaged by electrocoagulation during TUR) areas were omitted. The quantitation of MIB-1 score could be reliably carried out (the result of immunostaining was acceptable, there was no confounding background staining and the specimen was representative containing sufficiently cancer tissue) in 196/207 (94%) of cases. The method has been previously tested [32]. According to the study of Sallinen [32], two immunoreactivity levels (8% and 15%) were screened and the cut-off point of 15% was chosen.

p53 protein

p53 protein was detected by routine method as described above [19]. p53 expression (CM1 antibody, Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK; dilution 1:1200) was scored from the area which contained the highest fraction of positive nuclei. The fraction of positive cells was recorded and at least 2000 cells were evaluated. Bladder cancer biopsy specimen showing intense positivity for p53 was used as a positive control. A negative control was processed without primary antibody. The cut-off value for p53 was based on the study of Sarkis [33].

Bcl-2 protein

For immunohistochemical demonstration of bcl-2 protein, 5-μm sections from the primary bladder carcinomas were processed as described in connection with MIB-1 immunohistochemistry. After microwave heating the tissue sections were incubated with a monoclonal anti-bcl-2 protein (Dako, Denmark) antibody diluted at 1:400 in PBS. Several dilutions of the antibody were tested to avoid background staining and to find the optimal staining before the entire series was processed. Sections were washed twice for 5 min with PBS, and incubated for 20 min with biotinylated secondary antibody (Vector, California, USA) diluted at 1:200 in PBS. Sections were washed twice for 5 min with PBS, developed with diaminobenzidine tetrahydrochloride substrate (Sigma, UK), slightly counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted.

Scoring of bcl-2 protein expression

Twenty microscopic fields (× 250 magnification) were evaluated (at least 2000 cells). The immunoreactivity of bcl-2 protein in basal cells (one to three cell layers) was scored negative or positive. The immunoreactivity of bcl-2-protein in non-basal tumor cells was scored positive (1–2) or negative (0). The immunoreactivity of bcl-2 in positive cases was further classified as weak (1) or strong (2). The fraction of bcl-2-positive cells was also estimated. Histologically confirmed B-cell lymphoma biopsy specimens were used as positive controls. Tumor-infiltrating lymphocytes served as internal controls and a part of them was positive in all sections. Sections prepared without primary antibody were used as negative controls.

EGFr

The immunoreactivity of EGFr was detected by a polyclonal antibody (Santa Cruz, USA, cat: sc-03) and a dilution of 1:1000 was used. The sections were processed according to a routine method [19]. The sections were pretreated in 0.5% pepsin for 20 min (37 °C). Sections from normal human placenta were used as positive controls, whereas sections prepared without primary antibody were used as negative controls.

Scoring of EGFr immunoreactivity

The intensity of staining was graded subjectively into four categories by light microscopy: negative (0), weakly positive (1), equal to placenta (2) and stronger than placental control (3). The entire section was screened and the scoring was based on the findings in tumor areas with the strongest positivity for EGFr. The sections which showed weaker or equal positivity to human placenta were considered negative. All sections showing stronger staining than human placenta were considered EGFr positive in the final analysis. The scoring was based on the evaluation of the cytoplasmic and membrane staining together.

Statistical methods

The associations between bcl-2, p53, concurrent bcl-2/p53 and EGFr scores, tumor grade, stage and the expression of MIB-1 were assessed by Fisher's exact test, and two tailed P values were used. The following levels of significance were used: $P < 0.05$ significant and $P < 0.001$ highly significant.

Results

The clinical data and the distribution of patients in the pathological stages and WHO grades are shown in Table 1. Immunoreactivity of p53 protein was located in the nucleus. In the current study, the tumors were placed in two groups according to p53 immunoreactivity (< 20% positive tumor cell nuclei, ≥ 20% positive cell nuclei). The immunoreactivity of p53 (Fig. 1) correlated highly significantly with tumor grade (Table 2) and T category (Table 3). A clearly notable correlation was also found between p53 positive staining and MIB-1 immunoreactivity in pTa carcinomas (Fig. 2, Table 4). Fifty-nine percent of tumors with MIB-1 ≥ 15% were p53 positive, while only 25% of tumors with MIB-1 < 15% were p53 positive (Table 4).

In normal transitional epithelium adjacent to tumors, bcl-2 protein was usually immunostained in one to three basal cell layers. The immunoreactivity of bcl-2 protein in non-basal cells showed intratumor heterogeneity as regards staining intensity and fraction of positive cells. Immunoreactivity of bcl-2 protein was cytoplasmic, while also nuclear envelope in some of the tumors showed weak positivity and mitotic cells expressed bcl-2 protein. Bcl-2 immunostaining (Fig. 3) in non-basal cells of tumors correlated significantly with histological aggressiveness of the neoplasms (Table 2). Moderately and poorly differentiated tumors were more often positive for bcl-2 than were well-differentiated bladder cancers. A significant difference was also seen between grade 2 and grade 3 tumors. Between T category and bcl-2 immunostaining a noteworthy relationship was found (Table 3) as T1 bladder cancers were significantly more frequently bcl-2 positive than Ta tumors. A notable association between positive staining and expression of MIB-1 was also observed (Table 4). Thirty-nine percent of tumors with MIB-1 < 15% stained positively for bcl-2, whereas 64% of neoplasms with MIB-1 ≥ 15% were bcl-2 positive.

Simultaneous immunostaining of bcl-2 and p53 was highly significantly associated with tumor grade (Table 2) and stage (Table 3). Both markers were found to be positive in 10% of grade 1, in 29% of grade 2 and in 68% of grade 3 tumors. Seventeen percent of Ta tumors and 57% of T1 tumors showed simultaneous im-

Table 1 Clinical data and distribution of patients by pathological stage and WHO grade

Number of patients	185		
Mean age at diagnosis (years)	66 (range 30–89)		
Females/males	48/137		
Histological grade	Ta	T1	Total
1	88	–	88
2	53	19	72
3	7	18	25
Total	148	37	185

Fig. 1 p53 protein is expressed in almost all the nuclei in a WHO grade 3 transitional cell bladder cancer, × 400

Table 2 Immunoreactivity of p53, bcl-2, EGFR, MIB-1 and concurrent immunostaining with p53 and bcl-2 in grade 1, 2 and 3 tumors

	Grade 1 (*n*)	Grade 2 (*n*)	Grade 3 (*n*)	*P* value	
p53 < 20%	71	41	6	0.001	G1 vs G2
p53 ≥ 20%	17	31	19	0.005	G2 vs G3
				< 0.00001	G1 vs G3
bcl-2 –	64	29	4	0.00005	G1 vs G2
bcl-2 +	24	43	21	0.029	G2 vs G3
				< 0.00001	G1 vs G3
EGFR –	69	34	7	0.00006	G1 vs G2
EGFR +	19	38	18	0.10609	G2 vs G3
				< 0.00001	G1 vs G3
MIB < 15%	78	42	4	0.00002	G1 vs G2
MIB > 15%	10	30	21	0.00036	G2 vs G3
				< 0.00001	G1 vs G3
Nonconcurrent p53/bcl-2	79	51	8	0.00384	G1 vs G2
				0.00087	G2 vs G3
Concurrent p53/bcl-2	9	21	17	< 0.00001	G1 vs G3

Fisher's exact test for 2 × 2 tables, two-tailed P values

munoreactivity with p53 and bcl-2. Likewise, concurrent immunoreactivity of p53 and bcl-2 correlated with the proliferation activity of tumors. Only 14% of tumors with MIB-1 < 15% immunostained concurrently p53 and bcl-2, whereas 51% of tumors with MIB ≥ 15% showed positivity for both markers.

EGFr positivity was located in the cytoplasm and cell membranes. In low-grade tumors, weak immunoreactivity was usually confined to the basal cell layers, whereas in poorly differentiated tumors all cell layers showed immunolabeling with EGFr protein. Overall 41% of the tumors were classified as EGFr positive (75 out of 185) (Fig. 4). A significant relationship was observed between tumor category and EGFr positivity (Table 3). Thirty-seven percent of Ta cancers were positive for EGFr, while 65% of T1 tumors were EGFr positive, respectively. A significant association was also found with tumor grade (Table 2). Twenty-two percent of well-differentiated carcinomas were EGFr positive, whereas 53% of moderately, and 72% of poorly, differentiated carcinomas were positive for EGFr. A highly significant correlation with MIB-1 immunoreactivity was also established (Table 4). Twenty-eight percent of tumors expressing MIB-1 < 15% were EGFr positive, while 66% of carcinomas expressing MIB-1 ≥ 15% were positive for EGFr as well.

Table 3 Immunoreactivity of p53, bcl-2, EGFR, MIB-1 and concurrent immunostaining with p53 and bcl-2 in Ta and T1 tumors

	Ta (*n*)	T1 (*n*)	*P* value
p53 < 20%	106	12	0.00001
p53 ≥ 20%	42	25	
bcl-2 –	100	9	< 0.00001
bcl-2 +	48	28	
EGFR –	97	13	0.00125
EGFR +	51	24	
MIB-1 < 15%	118	11	< 0.00001
MIB-1 > 15%	30	26	
Nonconcurrent p53/bcl-2	122	16	< 0.00001
Concurrent p53/bcl-2	26	21	

Fisher's exact test for 2 × 2 tables, two-tailed *P* values

Table 4 Immunoreactivity of p53, bcl-2, EGFR and concurrent immunostaining with p53 and bcl-2 in relation to the immunoreactivity of MIB-1

Variable	MIB-1 (*n*) <15%	MIB-1 (*n*) >15%	*P* value
p53 < 20%	93	25	< 0.00001
p53 ≥ 20%	31	36	
BCL-2 –	75	22	0.00278
BCL-2 +	49	39	
EGFR –	89	21	< 0.00001
EGFR +	35	40	
Nonconcurrent p53/bcl-2	107	30	< 0.00001
Concurrrent p53/bcl-2	17	31	

Fisher's exact test for 2 × 2 tables, two-tailed *P* values

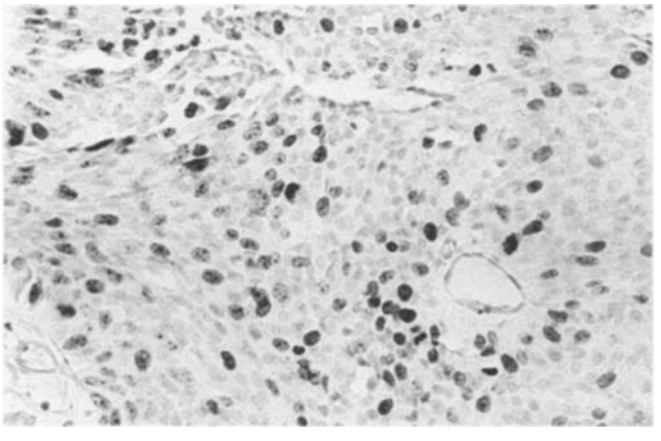

Fig. 2 A WHO grade 3 transitional cell bladder cancer where most of the nuclei are positive for MIB-1, × 400

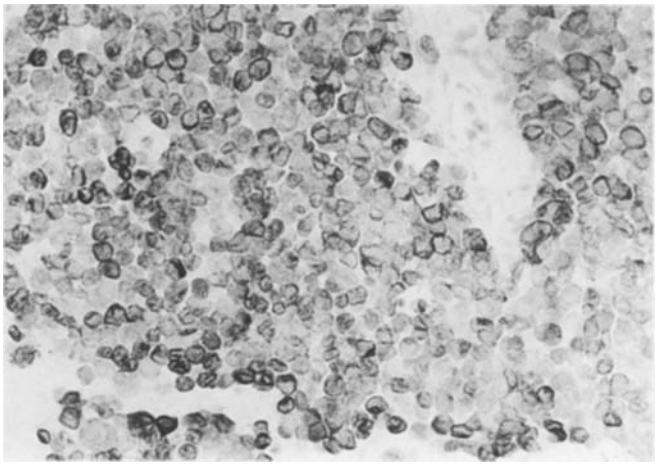

Fig. 3 Strong positivity for bcl-2 protein in the cytoplasm of transitional cells in a papillary grade 2 transitional cell carcinoma. Note bcl-2 positivity of the mononuclear cells in tumor stroma, x400

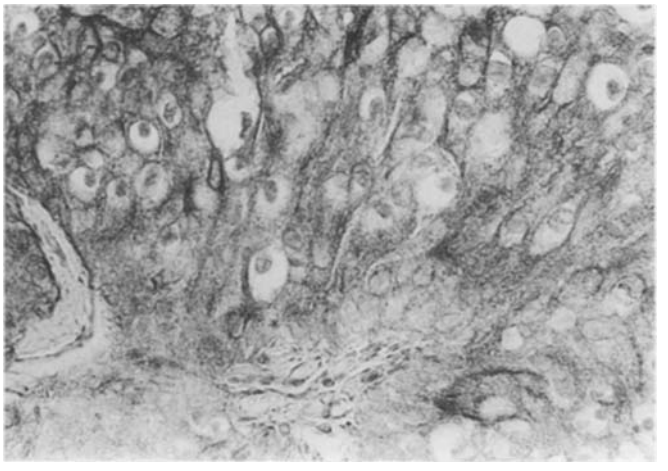

Fig. 4 Strong positivity for EGFR in a grade 2 transitional cell tumor. Immunopositivity is located on the cell membranes and in the cytoplasm of the cells, × 400

Discussion

p53 is a tumor suppressor gene coding for a cell-cycle-related DNA-binding protein [37]. Intact *p53* gene functions as a checkpoint during cell progression through the cell cycle [2]. Either the loss of normal p53 protein, or the presence of mutant p53 protein, may contribute to neoplastic cell growth [2, 8, 9, 13, 24]. The immunohistochemistry of p53 is a simple method and according to Esrig [8] it strongly correlates with mutations of the *p53* gene in bladder cancer. The mutated p53 protein can be detected by immunohistochemistry because of its considerably longer half-life than that of wild-type p53, wherefore it accumulates within the nucleus [8, 9]. Previous studies suggest that bladder cancers exhibiting overexpression of p53 protein can have a higher probability of disease progression [9, 33]. It is a general assumption that oncogenes and tumor suppressor genes are involved in the regulation of cell differentiation, cell death and control of cell proliferation. In a number of earlier studies the relationship between p53 expression and cell proliferation in various tumors has been studied [3, 14, 18, 28, 35, 42]. While some of the reports have shown an increased proliferation rate in p53-positive tumors [14, 18, 28, 35], such a correlation has not been established in all studies [3]. One of the most recognized methods for proliferation estimation is the Ki-67 antigen, the expression of which has been shown to have strong prognostic value [4, 10]. New monoclonal antibodies (MIB-1–3) [16] and an antigen retrieval method [5] have made it possible to assess Ki-67 expression in routinely processed paraffin blocks.

In the current study, the immunoreactivity of p53 correlated highly significantly with tumor grade and stage. Furthermore, p53 immunostaining correlated highly significantly with MIB-1 immunolabeling. However, in contrast to the results by Moch [24], we also found a difference in pTa carcinomas, whereas there was only a trend to be seen in the group of pT1 tumors. The variance in results may be due to the use of different cut-off values of p53 positivity. We also used a threshold value (</≥15%) for the MIB-1 immunostaining. The cut-off point was based on the study of Sallinen et al. [32].

Apoptosis or programmed cell death represents a mechanism by which cells possessing DNA damage can be deleted [45]. Experimental studies on bladder cancer suggest that apoptotic cell death is of importance during the process leading to invasive disease [38]. Evidence that wild-type p53 protein is important for induction of apoptosis in cells with a considerable amount of DNA damage resulting from chemotherapy or radiation is increasing [23]. Recently, wild-type p53 has been reported to play a role in the induction of programmed cell death by growth factor deprivation [46] possibly due to downregulating *bcl-2* [11]. The *bcl-2* proto-oncogene is a known inhibitor of apoptosis which was originally cloned from the breakpoint of a t(14:18) translocation present in many human B-cell lymphomas [39]. *Bcl-2* blocks a final common pathway for programmed cell death and, correspondingly, mutant p53 appears to inhibit apoptosis [22, 30, 43]. Clinically, bcl-2 expression is associated with a poor prognosis in several cancers [7]. Interestingly, in some malignancies bcl-2 immunoreactivity is, however, associated with a favorable outcome [15, 41]. Furthermore, it seems that the inhibitor of programmed cell death inhibits anticancer drug efficacy, and that classical pharmacological agents are powerless against it [7]. In colorectal tumorigenesis abnormal activation of the *bcl-2* gene appears to be an early event inhibiting apoptosis, and may thus facilitate tumor progression [36]. The development of bladder cancers from superficial papillary tumors to invasive carcinomas resembles the progression patterns found in colorectal neoplasias. At present, there are no published reports available on the prognostic significance and histopathological correlations of *bcl-2* gene in superficial bladder cancer.

In our series, tumors that invaded lamina propria, and tumors with increasing aggressiveness, were more often bcl-2 positive. Nevertheless, there also existed bcl-2-positive Ta and G1 tumors, and some of T1 and poorly differentiated carcinomas were bcl-2 negative, respectively. We furthermore observed that bcl-2-positive tumors were more often positive for MIB-1 than bcl-2-negative tumors. Piris et al.[29] in their study with high-grade B-cell lymphomas showed that simultaneous immunoreactivity of bcl-2 and p53 was associated with a poorer prognosis than that of p53 alone. In the current series, it could be observed that simultaneous immunoreactivity of p53 and bcl-2 was highly significantly associated with tumor grade, stage and proliferation activity. A difference between both well and moderately differentiated carcinomas as well as between moderately and poorly differentiated carcinomas was found.

Epidermal growth factor (EGF) is a 53-amino-acid peptide of 6 kDa that is a potent mitogen and widespread in human tissues [6]. The actions of EGF are mediated by binding to the external domain of a transmembrane receptor (EGFr). Bladder tumors also contain EGF receptors, and an association between EGFr positivity and tumor category has been shown [26]. Neal et al.[26] compared the prognostic value of EGFr with established factors (stage, grade, size and multiplicity) in a study including 52 superficial bladder carcinomas. EGFr positivity proved to be the best and, in fact, the only significant predictor of progression in superficial bladder cancers. In our study, a highly significant association between EGFr positivity and tumor stage was established. The T1 tumors were more often positive for EGFr than the Ta tumors. There was also a highly significant difference in the EGFr status between well- and moderately differentiated carcinomas, while there was only a trend to be seen between grade 2 and grade 3 tumors. Furthermore, we compared the EGFr status to MIB-1-immunostaining and found that tumors with < 15% MIB-1-positive nuclei were highly significantly more often EGFr negative than carcinomas containing

≥ 15% MIB-1-positive nuclei. The concomitant immunostaining of EGFr and MIB-1 suggests that EGFr immunoreactivity is related to the proliferation activity of cancer cells. This is in accordance with the results of Lipponen and Sauter [19, 34].

In conclusion, our results with 185 newly diagnosed SBCs relate the immunostaining of p53 and EGFr to tumor grade, stage and cell proliferation. We have not found previously published reports on the immunoreactivity of bcl-2 in superficial bladder cancers. Our study shows that immunostaining of bcl-2 is strongly associated with the histopathological aggressiveness, stage and proliferation activity of tumors. Furthermore, the simultaneous immunoreactivity of p53 and bcl-2 highly significantly correlates with tumor grade, stage and the expression of MIB-1. Our homogeneous cohort consists of three randomized treatment groups of equal size (TUR alone, TUR with a single dose interferon, TUR with a single dose of epirubicin). Whether these markers, even though they correlate very well with established prognostic factors, are able to predict progression with a better accuracy, remains to be seen after a sufficient follow-up period of current prospective material.

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