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Piotr Kaczmarek[1], Dariusz Tosik[2], Ewa Majewska[3], Zbigniew Baj[1]

Quantitative Analysis of Cyclin E and Protein p34 cdc2 Expression in Superficial Bladder Cancer*

[1]Department of Pathophysiology and Clinical Immunology,
[2]Department of Anatomy and Histology,
[3]Department of Pathophysiology and Immunopathology of Effort, Medical University of Łódź

In the present study, the expression of cyclin E and kinase p34 cdc2 was investigated in preinvasive bladder tumors. The study material consisted of bladder sections (grades: GI – 16 cases, GII – 10, and GIII – 12) collected from 38 patients in the course of the tumor electroresection. Immunohistochemical examinations were carried out with immunoperoxidase method. Antigens were labeled with NCL-CYCLIN E or NCL-p34 cdc2 monoclonal antibodies (Novocastra, UK). Positive reaction was demonstrated using ABC-universal Kit (Novocastra, UK). Differences in the protein expression in relation to the tumor grade were determined with a non-parametric Mann-Whitney's test. Increasing grade of tumors was associated with down regulation of cyclin E visible as lower percentage of cyclin E-positive cells. These changes were statistically significant for GI group as compared to groups GII and GIII ($p<0.001$). There were no differences between the study groups in the p34 protein expression. Cyclin E expression was inversely correlated with tumor grade therefore may be helpful in establishing therapeutic procedure.

Introduction

Bladder carcinoma is the fifth neoplasm as regards the frequency of incidence and the twelfth as regards the number of deaths caused by neoplastic diseases [16]. The patients' age also decreases [2].

In recent years, there has been significant advancement in studies on molecular and genetic mechanisms of neoplasm development. Much attention is paid to the disturbances in cell cycle, the abnormal course of which may lead to neoplastic cell transformation [6, 15].

The presence of internal control points is an important element of cell cycle regulation. Normal regulation of the passage through the points on G1/S and G2/M phases boundary is of particular importance [10, 16]. Recent studies have demonstrated that the damage of mechanisms controlling the course of phase G1, in which cell proliferation and differentiation is initiated as well as passage from phase G1 to S plays the most important role in the initiation of neoplastic transformation [1, 5, 7].

The analysis of expression of chosen cell cycle related proteins and the individual case histories may give us a helpful tool being a predictive and prognostic marker in TCC patients.

This study was aimed at determining the relation between the level of cyclin E and protein p34 (cdc2) expression and the grade of tumor differentiation estimated by a histopathologist.

Material and Methods

The study material consisted of bladder tumor sections obtained from 38 patients in the course of transurethral resection (TUR). The investigated group included 14 women and 24 men, aged 48–76 years, mean 63.8 years. All neoplasms were diagnosed as transitional cell carcinoma. The tumor staging and grading were determined by pathologist according to International Union against Cancer and World Health Organization classifications. There were 16 specimens of grade GI, 10 of GII and 12 of GIII. According

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to clinical classification all tumors belonged to pTa group (preinvasive carcinoma, where the tumor grows only exophytically).

Immunohistochemical examinations

Immunohistochemical examinations were performed with the use of immunoperoxidase method. Tissues were fixed in 10% buffered formaldehyde for 24 hours in 4°C, and then embedded in paraffin. Tissue sections 4 m-thick were placed on silicone slides and then dewaxed in xylene and hydrated in decreasing concentrations of ethanol.

Endogenous peroxidase activity was inhibited with 3% H_2O_2 in methanol for 10 min at room temperature. Antigens were exposed by heating specimens in a microwave oven in phosphate buffer, pH=6.0, for 7 min at 730 W, then for 5 min at 350 W. The sections were rinsed in TBS (TRIS Buffered Saline – Sigma USA) pH=7.6, three times for 5 min. Non-specific staining was blocked with non-immune mouse serum for 20 min. The antigens were detected with NCL-CYCLIN E and NCL-p34 cdc2 monoclonal antibodies (Novocastra, UK) with which sections were incubated for 60 min at 25°C. Concentrations of antibodies were matched experimentally to obtain the best reaction. The dilution of primary antibodies was: 1:60 for cyclin E and 1:200 for p34 cdc2. The positive reaction was demonstrated using ABC-universal Kit (Novocastra, UK): biotinylated anti-mouse secondary antibody (30 min at room temperature) and then avidin and biotinylated peroxidase. DAB was used as chromogen (Sigma, USA). Finally, the specimens were stained with hematoxylin and embedded in DPX (Fluke CH).

Quantitative analysis

In the case of cyclin E detection, the cells with stained nucleus were qualified as positive. Nuclear-cytoplasmic staining was characteristic for cdc2 kinase. The investigated protein expression was estimated as the percentage of positive cells /1000 cell examined in 15 randomly selected microscopic fields at 400× magnification. Quantitative analysis was carried out with the IMAL-512 image analysis system. The tresholding was performed interactively depending on internal standard of grey level. According to the percentage of positive cells each specimen was qualified to one of three groups: O – no labeling; (+) <20% of positive cells; (++) >20% of positive cells.

Statistical analysis

Differences in protein expression in relation to tumor grade were determined with non-parametric Mann-Whitney's test at the level of significance $p<0.01$.

Results

Positive staining for cyclin E was present in 76% of specimens, irrespective of tumor grade.

Quantitative analysis of cyclin E expression demonstrated a gradual decrease in the percentage of positive cells with an increase in the neoplasm grade (Table 1). This decrease was statistically significant for group GI as compared to groups GII and GIII ($p<0.001$). No statistically significant changes were found between neoplasms moderately and poorly differentiated (GII and GIII) (Fig. 1).

TABLE 1
The dependence of cyclin E expression on tumor grade

Tumor grade	Number of cases	Positive cells mean SD [%]	Specimen staining intensity [%]		
			0	+	++
GI	16	47.1 1.9	8.3	58.3	33.4
GII	10	20.5 2.9*	22.2	77.8	0
GIII	12	18.1 3.1*	60	40	0

* $p<0.001$ vs GI

TABLE 2
The dependence of protein p34 cdc2 expression on tumor grade

Tumor grade	Number of cases	Positive cells mean SD [%]	Specimen staining intensity [%]		
			0	+	++
GI	16	31.89 7.55	16.7	58.3	25
GII	10	33.23 6.98	22	56	22
GIII	12	32.74 8.01	10	60	30

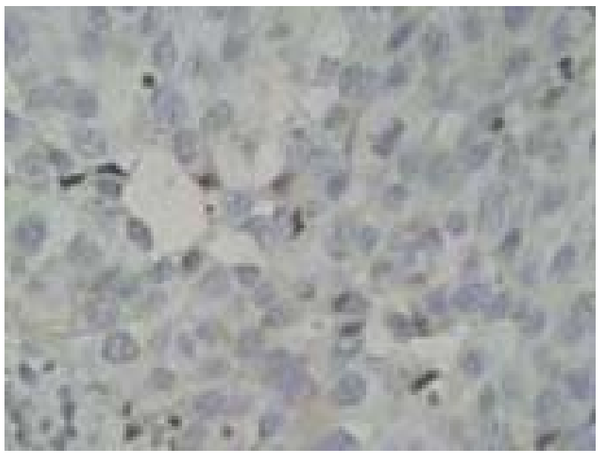

Fig. 1. Expression of cyclin E in urothelial carcinoma.

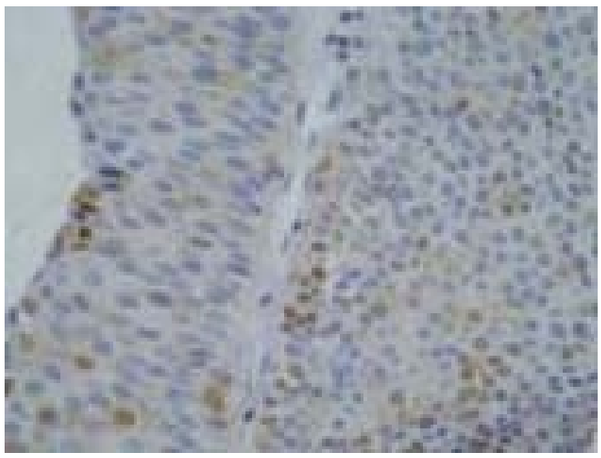

Fig. 2. Expression of p34 protein in urothelial carcinoma.

The percentage of positive cells with protein p34 expression was at the medium level of 32.62%, and no significant differences were observed between the investigated groups (Table 2, Fig. 2).

Discussion

Recently, disturbances in the cell cycle have been one of the main directions of studies on the process of carcinogenesis. Cell cycle is a complex process and about 100 genes are responsible for its course. Transition into particular cycle phases is monitored by specific mechanisms called control points [3]. Specific proteins control preparation and readiness for transition into the consecutive phase of the cell cycle.

In our studies cyclin E expression was found in 76% of all cases examined. The percentage of positive cells decreased with an increase of tumor grade. Similar results were obtained by Kamai and co-workers who carried out their studies on 145 TCC cases [5]. They found inverse correlation between cyclin E expression and the proliferative index (Ki-67) and histopathological parameters. Opposite results were noted by Osman et al. [13] in 55 TCC cases, were no significant correlation between cyclin E expression and clinical data or proliferative index were found. The same results were noted by Khan et al. [8].

However, Richter and co-workers investigating almost 2000 cases of bladder tumors, observed a significant increase in cyclin E expression along an increase in the histopathological grade [14]. Similar results were noted by Makiyama et al. [11]. Studies on cyclin E location in the cell during cell cycle, performed by Juan and Cordon-Cardo [4] on isolated lines of bladder cancer cells demonstrated its presence in nuclear structures only in the late G1 phase and in the course of transition from G1 to S. However, in some lines, the cyclin was present in nucleus in all the cycle phases. The authors suggest that nucleus mediates the transport of cyclin E from nucleus to the cytoplasm, where it undergoes enzymatic degradation. In some types of bladder carcinoma, this mechanism controlling the level of cyclin is disturbed. Kinase p34 together with cyclin B are responsible for the cell transition through G2/M restriction point and for normal course of mitosis. Decrease in p34 level leads to cell cycle cessation in phase G2 and to initiation of repair mechanisms, most frequently of apoptosis [9, 12]. In our studies p34 expression was independent on the tumor grade.

Conclusions

We suggest that a patient with diagnosed bladder carcinoma of the grade GI should be subjected to similar standards of treatment as a patient with diagnosed neoplasm of the grades GII or GIII. This analysis concerns mainly the continuation of treatment by the immune system modulation method with the help of Bacillus Calmette-Guerin Suspension (BCG).

It seems that the analysis of cyclin E expression cannot be the base for determination of the disease advancement however; it may be helpful, in some pathological cases, in establishing the therapeutic procedure.

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Address for correspondence and reprint requests to:
Piotr Kaczmarek
Department of Pathophysiology and Clinical Immunology
Medical University
pl. gen. J. Hallera 1
90–647 Łódź
E-mail**:** pkacz@neostrada.pl