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ORIGINAL ARTICLE

Immunohistochemical detection of cyclin E in transitional cell carcinoma

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Summary

Purpose: *It is known that expression disorders of cell cycle regulators play an important role in the development and prognosis of various malignant tumors. Cyclin expression changes during the cell cycle. This work aimed to analyse the expression of cyclin E in transitional cell carcinoma (TCC) and also to compare the expression of cyclin E with tumor stage and histological grade as well as to determine possible existence of differences in the expression of cyclin E in TCCs of the upper and lower urothelium.*

Methods: *Twenty-four cases of TCC of the urinary tract were retrospectively analysed (6 cancers of the renal pelvis, 2 of the ureter and 15 of the bladder; 4 were infiltrative). Immunohistochemical staining for cyclin E of the analysed transitional cancer cells was assessed semiquantitatively: diffuse cyclin E expression + + + (> 50% of all cells), expression in larger groups of cells: + + (up to 50% of all cells), expression in individual cells or small cell clusters: + (<10% of all cells), and absence of expression. Tumor stage was based on clinical and morphological criteria. WHO classification (Lyon 2004) was used for determination of the histological grade.*

Results: *Non-parametric Spearman's correlation showed that there was no statistically significant correlation between tumor stage and expression of cyclin E ($\rho = -0331$, $p > 0.05$). Also, no statistically significant correlation between grade and the expression of cyclin E ($\rho = -0077$, $p > 0.05$) was found. x^2 test results showed no statistically significant difference ($x^2 = 2.136$, $p = 0.775$) in the expression of cyclin E between upper and lower urothelium.*

Conclusion: *This study showed non significant decreased expression of cyclin E with poor differentiation, muscle invasion and upper/lower urothelium. Expression of cyclin E decreased with increasing histological grade and stage of the tumor.*

Key words: cyclin E, immunohistochemistry, transitional cell carcinoma

Introduction

TCC is the 7th most common malignancy in humans (3.2%) [1]. In about 95% of the cases the tumor arises in the bladder, and in the remaining 5% it is detected in the ureter or pyelocalyceal system [2]. Depending on whether TCC breaks through the basement membrane or not, it is divided into invasive and noninvasive. Noninvasive tumors have a very good prognosis, but are relatively rare. At the time of diagnosis most tumors are usually invasive and bear a worse prognosis. Five-year survival is < 60% in stages II and III and < 10% in stage IV.

Cell cycle is a highly organized and complex process that allows a complete and accurate replication of cells before their division. Cyclin, cyclin dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CDKIs) play a key role in regulating the cell cycle [3-5]. Cyclin E is the product of a chromosome gene 19q12-Q13 [6] and is expressed in late G1 phase of the cell cycle. Its role is to facilitate the entry of cells into S phase. It is a 395 amino acid protein which contributes to a normal cell proliferation and development. Its abnormal expression may accelerate the G1 phase of the cell cycle [7]. It forms a complex with CDK2 (cyclin dependent kinase 2) and participates in the phosphorylation of retinoblastoma gene product (pRb). In the active form pRb (retinoblastoma protein) is related to the

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E2F transcription factor, preventing the transition of cells from G1 into S phase of the cell cycle. Loss of pRb function is believed to promote abnormal proliferation as a result of the deregulation of the E2F transcription factor, followed by increased expression of the S-phase specific genes [8-10].

Cyclin expression changes during the cell cycle. Due to the discovery of abnormal cyclins expression in cancer, it is assumed that some of them are involved in oncogenesis, acting as oncogenes [3]. Loss of expression of cyclin E occurs in prostate, breast, colorectal and esophageal cancers.

This work aimed to analyse the expression of cyclin E in TCC and also to compare the expression of cyclin E with tumor stage and histological grade, as well as to determine possible differences in the expression of cyclin E in cancers of the upper and lower urothelium.

Methods

The study was performed after approval of the Institutional Ethics Committee. Twenty-four cases of TCC of the urinary tract were analysed. Disease stage was based on clinical and morphological criteria and WHO classification (Lyon 2004) was used for the determination of the histological grade.

Grade III disease was found in 13 patients, grade II in 9, and grade I in 2. Stage I was found in 10 patients, stage II in 7, stage III in 3 and stage IV in 4. The study included 6 cancers of the renal pelvis, 2 of the ureter and 15 of the bladder, of which only 4 were invasive (3 cases infiltrating the submucosa and 1 case infiltrating massively the muscular layer).

The bioptic material was stained immunohistochemically. For unmasking of antigens the specimens were processed in a microwave oven in citrate buffer (pH 6.0) with 3 cycles of 5 min each. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min. In order to reduce nonspecific staining we used normal, nonimmune pork serum dilution 1:10 for 30 min. Then, cyclin E primary antibody (M-20, Santa Cruz, USA), dilution 1:400 for 60 min was used. Staining was performed by streptavidin-biotin technique using DAKO LSAB + kit. As a chromogen 3.3 diaminobenzidine (DAB) was utilized, and for contrast staining Mayer's hematoxylin.

Immunohistochemical staining of the analysed transitional cancer cells was determined semiquantitatively. Assessment was made as follows:

a) diffuse expression (> 50% of all cells): + + +

b) expression in larger groups of cells (up to 50% of all cells): + +

c) expression in individual cells or small cell clusters (< 10% of all cells): +

d) absence of expression (Figure 1 A-D).

Cases with expression in larger groups of cells and diffuse expression were classified into one group. These cases were considered to have intact (normal) expression of cyclin E since normal urothelium showed diffuse cyclin E expression. Cases with expression in individual cells or small cell clusters and cases with absence of expression were classified into a second group and regarded as having decreased cyclin E expression.

Statistical analysis

Data processing was performed using methods of descriptive statistics and non-parametric Spearman's correlation test (95% confidence intervals / 95% CI) and x^2 test with $p<0.05$ considered as statistically significant.

Results

Of 24 patients with TCC 20 (83.3%) were males and 4 (16.7%) females. Their average age was 63.79 ± 11.9 years (range 36-83).

TNM tumor stage is shown in Figure 2. The most frequent stage was stage I (10 cases, 41.7%). Stage II included 7 patients (29.2%) and stage IV 4 (16.7%). Stage III had the lowest incidence (n=3, 12.5%).

Tumor grade III was most common, found in 13 patients (54.2%). Second in the row was grade II (9 patients, 37.5%), while grade I was found in 2 patients (8.3%; Figure 3).

In 20 patients (83.33%) cyclin E expression was reduced or absent. Only 4 cases (16.67%) showed diffuse and moderate expression. No statistically significant correlation was found between tumor stage and cyclin E expression ($\rho = –0331$, $p> 0.05$). Preserved expression of cyclin E was observed in 37.5% of the cases (9/24). Negative expression of cyclin E was observed in 20.83% (5/24) of stage II disease cases. Cyclin E expression decreased with increasing disease stage (Table 1).

No statistically significant correlation concerning grade and the expression of cyclin E was registered ($\rho = –0077$, $p> 0.05$). Reduced expression of cyclin E was observed in 84.62% (11/13) of the grade III cases. With increasing histological grade the loss of cyclin E expression became more visible (Table 2).

Also, no statistically significant difference ($x^2 = 2.136$, $p = 0.775$) in the expression of cyclin E of the upper (renal pelvis, ureter) and lower (urinary bladder, urethra) urothelium was noticed.

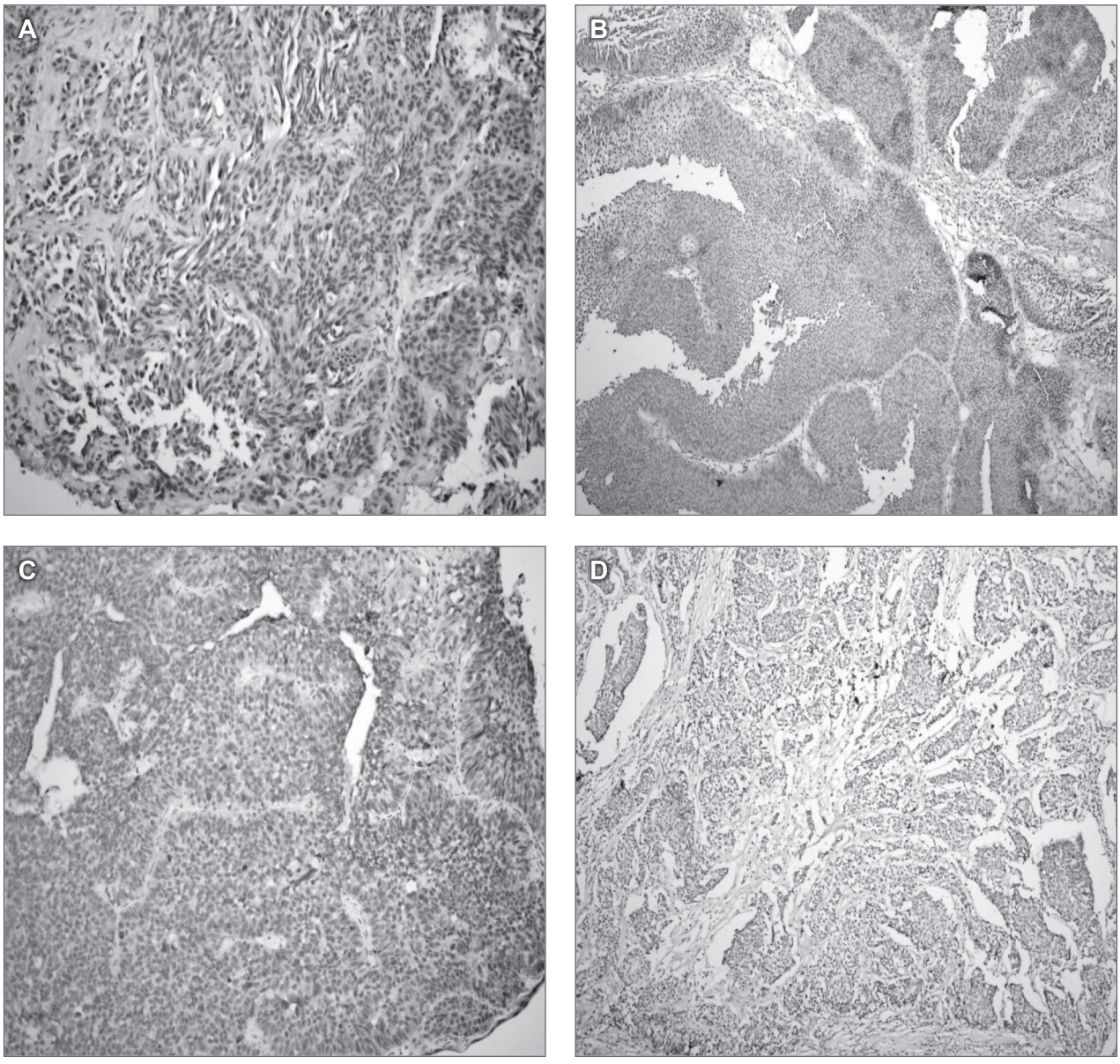

Figure 1. Immunohistochemical expression of cyclin E in transitional cell carcinoma. **A:** Stage I - Positive expression of cyclin E (>50% of positive cells). **B:** Stage II - Focally positive expression of cyclin E (<10% of positive cells). **C:** Stage III - Heterogeneous cyclin E expression (10-50% of positive cells). **D:** Stage IV - Negative cyclin E expression.

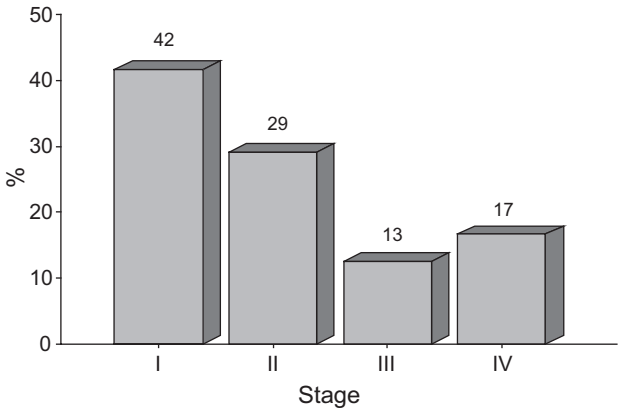

Figure 2. Tumor stage of 24 patients.

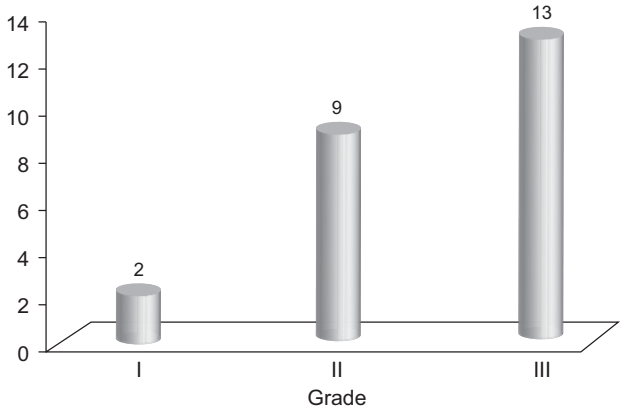

Figure 3. Tumor grade of 24 specimens.

Table 1. Relationship between stage of transitional cell carcinoma and cyclin E immunoexpression

Tumor stage / immunoexpression	*I n (%)*	*II n (%)*	*III n (%)*	*IV n (%)*	*Total n (%)*
+++	0 (0)	1 (14.3)	0 (0)	0 (0)	1 (4.1)
++	5 (50)	1 (14.3)	1 (33.3)	0 (0)	7 (28.7)
+	1 (10)	0 (0)	0 (0)	0 (0)	1 (4.1)
–	4 (40)	5 (71.4)	2 (66.6)	4 (100)	15 (61.5)
Total	10 (100)	7 (100)	3 (100)	4 (100)	24 (100)

Table 2. Relationship between histological grade and cyclin E immunoexpression

Histological grade / Immunoexpression	*I n (%)*	*II n (%)*	*III n (%)*	*Total n (%)*
+++	0 (0)	0 (0)	1 (7.7)	1 (4.17)
++	1 (50)	1 (11.1)	1 (7.7)	3 (22.5)
+	0 (0)	2 (22.2)	3 (23.1)	5 (20.8)
–	1 (50)	6 (66.6)	8 (61.5)	15 (62.5)
Total	2 (100)	9 (100)	13 (100)	24 (100)

Discussion

Cyclins are a group of regulatory proteins involved in cell cycle control performing their function by creating a complex with CDKs [3-5] regulating the activity of CDKs, and activating them when they reach a certain concentration in the presence of cyclin activating kinase (CAK). To maintain the exact sequence of events in the cell cycle, a single pair of CDK must act to specific control points. So far, we are familiar with 10 classes of cyclins, designated by letters of the alphabet from A to J. Cyclins are grouped into the G1 cyclins, such as cyclin E which controls the transition from G1 to S phase and into mitotic cyclins, such as cyclin B, which controls mitosis [11].

CDK activation by cyclin is closely regulated, preventing a permanent cell replication. This is achieved through CDKIs. These inhibitors prevent the effect of accumulated cyclin-CDK complex. CDKIs work on specific areas known as "place checking". There are two CDKI families: the first family (CIP / KIP) contains 3 proteins: p21, p27 and p57, which have an effect on many CDKs; the second family contains 4 proteins: p15, p16, p18 and p19, which exert selective effects on CDK4 and CDK6 during G1 phase [12,13].

Whether the cells continue to proliferate or halt their growth and differentiation is determined by numerous factors acting during the G1 phase of the cell cycle. The entry of cells into S phase depends on the coordinated activities of serine / threonine kinase CDK, which plays an important role in the G1 phase. Regulation of their function is crucial for proliferation and differentiation.

Dysregulation of the cell cycle can lead to cancer. Cyclins are positive regulators of the cell cycle. Many studies proved that overexpression of cyclins is linked to accelerated proliferation and cancer formation.

According to literature data, cyclin E expression in various types of tumors including TCC may be interpreted as a positive and a negative prognostic factor for relapse and survival [14]. Dobashi et al. [15] as early as 1998 suggested that the well differentiated cells of lung cancer, which had a low proliferative activity also had a strong expression of cyclin E. On the basis of these results they have suggested that cyclin E plays an essential role in cellular differentiation. Other studies, however, consider cyclin E as negative prognostic factor for survival in TCC [14]. We observed that cyclin E had a tendency to be expressed in invasive tumors and lymph node metastases. Several authors [14,15] showed that expression of cyclin E in embryonal testicular tumors was related to tumor stage and assumed that its presence is required for dissemination in the lungs. Donnellan et al. [16] showed that cyclin E was an adverse prognostic marker in TCC of the renal pelvis and ureter. Numerous studies have shown that disrupted or decreased expression of cyclin E in bladder cancer is an adverse prognostic factor for survival [14-16].

Many authors have evaluated the expression of cyclin using the classification originated from the works of Del Pizzo et al. [17], and Makiya and associates [18]. They placed TCCs that showed similar immunohistochemical staining as the normal tissue into the group with normal, i.e. preserved expression, and into the group with decreased expression they included TCCs that had both positive and negative staining areas. Ac-

cording to their research, as well as according to other authors [19-21], cyclin E showed a uniform expression in normal epithelium. Therefore, according to their criteria, preserved expression of cyclin E existed if > 50% of cells were stained, i.e. when the epithelium looked normal. And if stained cells were < 10% there was decreased expression of cyclin E.

In our sample, only 4 cases (16.67%) displayed a moderate or diffuse expression of cyclin E (+ + or + + +). As already said, these results are considered as normal, i.e. the expression of cyclin E was considered preserved in the investigated cases. In 5 cases (20.83%) a decreased expression of cyclin E was observed. And 15 cases (62.5%) were negative to antibodies to cyclin E.

Many authors tried to point out the connection between cyclin E expression with tumor stage and grade, lymph node invasion or metastasis in distant organs [17,19]. All of them proved that low expression of cyclin E was associated with poor differentiation, muscle invasion, lymph nodes metastasis and short survival [17,19,22].

In our study, cyclin E expression was preserved in 50% (5/10) of stage I patients, and was decreasing with increasing stage. By statistical processing we showed that there was no statistically significant correlation between tumor stage and expression of cyclin E, which is in accordance with the relevant literature data. Literature data suggested that normal expression of cyclin E existed in most cases of T1 tumors and decreased in T3 tumors, and that the level of cyclin E decreased with increasing tumor stage. Several investigators in their research also found no statistically significant association between abnormal expression of cyclin E and tumor stage [17-23].

Our research showed that the expression of cyclin E was preserved in 16.67% (4/24) of the cases, while in most cases (45.83%; 11/24) of histological grade III the expression of this cyclin was reduced. It was determined that the expression of cyclin E decreased with increasing histological grade. All previous studies have shown that a reduction in nuclear immunoreactivity of cyclin E occurred with increasing tumor grade [17-23]. Our results were consistent with the results of previous research. However, in our study we found that there was no statistically significant correlation between the grade of TCC and decreased expression of cyclin E, while other studies have shown statistical significance. This discrepancy could be explained by the small number of cases that we examined in comparison to the number of cases that have been included in other studies.

The explanation of the abnormal expression of cyclin E could be that in G1/S phase p16-cyclin D - CDK - Rb gene contributed to the development of the tumor. The deletion of p16, p21, cyclin D1 and cyclin E-CDK2 complexes were included in G1/S transition, by regulating the phosphorylation of Rb. In addition, p27 and cyclin E played a central role in the transition from late G1 to S phase. Considering the above speculations, we could say that the abnormal expression of cyclin G1 was a possible mechanism that allowed tumor cells to divide in an uncontrolled manner.

According to the results of cyclin E expression, it can be concluded that loss of expression was found in tumor tissue. Significant loss or reduction of expression of cyclin E was connected with increase in histological grade and pathological stage. Low expression of cyclin E was linked to poor differentiation, invasion, metastasis and shorter survival.

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