Cyclin A Overexpression in Carcinoma of the Renal Pelvis and Ureter including Dysplasia: Immunohistochemical Findings in Relation to Prognosis

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ABSTRACT

Several in vitro studies have shown that cyclin A gene alteration in the cell cycle plays an important role in carcinogenesis. We immunohistochemically examined the expression of cyclin A protein in 120 patients with transitional cell carcinoma (TCC) of the renal pelvis and ureter, including adjacent dysplastic lesions to determine their significance for the tumor behavior and patient prognosis. Cyclin A immunostaining of the nucleus was observed in 29 tumors (24.2%). Furthermore, 17 cyclin A-positive tumors (58.6%) had dysplastic lesions positive for cyclin A antibody. The prevalence of cases exhibiting cyclin A staining was higher in the high grade (P < 0.01) and invasive tumors (P < 0.05) than in the other types of tumors. In the selected 117 cases, patients whose TCCs expressed a high level of cyclin A protein had a significantly poorer prognosis than those without cyclin A expression (P < 0.01). These in vivo findings provide the first evidence for frequent and redundant cyclin A overexpression in TCC and suggest that cyclin A overexpression is related to the tumor behavior and patient prognosis. In addition, our observations indicate that overexpression of cyclin A may be one of the early events, at least in some cases, in the carcinogenesis of TCC.

INTRODUCTION

Determination of the sequences of molecular events leading to and following deregulation of the cell cycle control is crucial to our understanding of the development and progression of malignant tumors. Human cyclins, such as A type, B type, and G1 (C, D, and E) type cyclins, are prime cell cycle-specific regulators (1), and they play an important role in the control of major checkpoints of the cell cycle (2, 3). With the discovery of inappropriate expression of cyclins in some tumors, it has now been specifically hypothesized that some cyclins are intimately involved in oncogenesis, acting as proto-oncogenes (2, 3). Diverse patterns of redundant expression of particular cyclins, such as cyclins D1 and E, in different tumor cell lines have previously been reported in oncogenesis (4–14). A relationship of cyclin A abnormalities to carcinogenesis has also been noticed. Human cyclin A gene at a site of hepatitis B virus DNA integration was identified in a hepatocellular carcinoma in association with abnormality of the cyclin A gene (15, 16). Cyclin A also associates with adenovirus E1A oncoprotein (17, 18) and has been identified in complexes containing the E2F transcription factor and p107, which is structurally related to the retinoblastoma protein (18–22). Although little is known concerning the significant role of cyclin A in tumorigenesis, these studies suggest that cyclin A may be of value in identifying proliferative tumor cells, and its alteration may be an important step in the pathogenesis of certain cell lines and primary tumors.

There is little data available concerning whether premalignant states, including dysplasia, in the transitional epithelium progress to TCC. However, most reports have indicated that dysplasia acts as a common precursor to TCC because it shows a high propensity to progress to carcinoma in situ (23, 24). In addition, the presence of dysplasia adjacent to TCC is associated with an increased risk of subsequent recurrence and progression (25). Therefore, the features of dysplasia provide ideal systems to study the proposed multiple-step nature of carcinogenesis. Molecular and histological studies of preinvasive urothelial lesions have frequently demonstrated genomic and pathological alterations in carcinoma in situ and dysplastic lesions, suggesting that at least some of these abnormalities occur very early in oncogenesis (26, 27). Recently, increased expression of cyclin D1 protein was reported in precancerous lesions of colorectal mucosa (28). These findings prompted us to study the level of expression of cyclin A protein at different stages along the multistage process of urothelial carcinogenesis from normal mucosa to dysplasia and carcinoma.

In the present study, we extended these observations of cyclin A to in vivo conditions in human TCC to elucidate the potential role of the expression of cyclin A in tumor development and to assess their prognostic value, also examining the dysplastic epithelium adjacent to the tumor. The relationship with various clinicopathological factors was then analyzed.

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2 To whom requests for reprints should be addressed. Phone: 0888-66-5811; Fax: 0888-80-2336.

1 The abbreviation used is: TCC, transitional cell carcinoma.
MATERIALS AND METHODS

Patients and Tumor Samples. One-hundred twenty cases of human TCCs of the renal pelvis and ureter, which were all TCC cases obtained by radical nephroureterectomy between October 1981 and January 1997 at the Department of Urology of Kochi Medical School, the Division of Urology of both Kochi Takasu Hospital and Fujisaki Hospital, were retrospectively studied using immunohistochemistry. Tumors were processed in a similar fashion at all three institutions. Histological or clinical classification of tumors was performed according to the “General Rules for Clinical and Pathological Studies on Renal Pelvic and Ureteral Cancer (1990)” (29). Tumor specimens were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. In each case, all of the available H&E-stained sections were reviewed, and a representative block was chosen for further studies. There were 67 renal pelvic cancers and 53 ureteral cancers, including 10 cases with renal pelvic and ureteral cancers. Of 120 cases with TCCs, 21 have bladder cancer concurrent with renal pelvic or ureteral cancer. The patients included 84 men and 36 women, ages 45-93 years (median age, 71 years).

Immunohistochemistry with Cyclin A Antibody. Five μm-thick sections from archival formalin-fixed, paraffin-embedded tissue were placed on poly-L-lysine-coated slides (Sigma Chemical Co., St. Louis, MO) for immunohistochemistry. Cyclin A protein expression was assessed by immunohistochemical examination (streptavidin-biotin complex procedure) with a monoclonal antibody, Cyclin A (NCL-CYCLIN A, 6E6, IgG1; dilution, 1:50; Novocastra Laboratory, Inc., Newcastle upon Tyne, United Kingdom).

Deparaffinized tissue sections were placed in 10 mm citrate buffer (pH 6.0) and heated to 132 °C in an autoclave for 20 min for antigen retrieval, as shown in our previous study (27). After blockage of endogenous peroxidase activity with methanol containing 0.3% H2O2 for 30 min, the sections were incubated at 4 °C overnight with the monoclonal antibody to cyclin A protein. After washing with 0.1 M PBS (pH 7.4), the streptavidin-biotin complex (ABC) procedure was performed by using a streptavidin-biotin complex peroxidase kit (DAKO LSAB kit; Dakopatts, Kyoto, Japan) and by following the directions in the kit manual. Finally, slides were counterstained with methyl green. Positive or negative controls included in each experiment were run in parallel; these included replacement of the specific or nonspecific mouse IgG1 antibodies with PBS. The experiment was repeated, yielding essentially identical patterns of cyclin A distribution in each instance in each tumor specimen.

Tumor sections exhibiting definite staining of tumor cell nuclei with cyclin A antibody were scored as cyclin A positive. A visual assessment was made in such cases of the number of positive tumor cells as a proportion of the total expression of cyclin A protein as follows: negative cases (0%, or variable weak positivity in tumor cells: 0–10%); positive cases consisted of two types, one with heterogeneous (variable positivity in tumor cells: 10–60%) and the other with homogeneous (diffuse strong positivity in tumor cells: >60%) immunostaining.

Statistical Analysis. The correlations between the expression of cyclin A protein and the various clinicopathological factors considered were determined using the χ2 test at the 5% level.

Association between Cyclin A Expression and Prognosis. Survival was calculated from operation to the date of death or the date of the last follow-up (either a clinical visit or a discussion with the patient’s referring physician). Of all 120 patients selected, excluded from the follow-up studies were 3 patients (2.5%), who did not have at least 6 months of follow-up or who did not survive at least 6 months after operation. In the remaining 117 patients, all were clinically followed up more than 6 months, and there were no patients with inadequate follow-up. Median follow-up was 3.4 years (range, 0.5–10.5). At last follow-up, 66.2% of the patients were alive.

Analyses of survival data were performed using survival curves and the Kaplan-Meier method and log rank test. In addition, the Cox proportional hazards model was used to calculate and estimate the postoperative survival rate and to determine the significance of each prognostic factor used in histological or clinical classification. For multivariate analysis, variables were selected on condition that they were statistically significant and were only poorly correlated with each other.

RESULTS

Immunohistochemistry with Cyclin A Antibody. Cyclin A antibody immunoreactivity was homogeneous in 16.7% (20 of 120) of the TCCs. A certain degree of heterogeneous staining in the neoplastic cell population of immunoreactive cases was observed in 7.5% (9 of 120) tumors. Staining was predominantly observed in the nuclei (Fig. 1). Mitotic figures were found in cyclin A-positive tumors (Fig. 1, arrows). Of the tumors regarded as negative, 14 showed very weak traces of nuclear staining. In the present study, dysplastic lesions adjacent to carcinomas were observed in 101 cases (101 of 120; 84.7%), including cyclin A-positive 29 tumors. Furthermore, cyclin A expression was frequently detected in dysplastic lesions adjacent to 17 cyclin A-positive TCCs (17 of 29; 58.6%; Fig. 2, A-C). In the remaining 72 cyclin A-negative tumors with dysplasia, negative immunoreaction for cyclin A was also found in...
dysplastic lesions. In the normal tissues of the urinary tract, cyclin A immunoreactivity was restricted to the subsets of cells of the basal layer in the transitional epithelium of the urinary tract. ×250.

**Fig. 2** A. positive nuclear staining with anti-cyclin A antibody was detected in cancer cells (TCC, grade 2 > 1: arrow) as well as dysplastic cells (arrowhead; ×100). B and C, high magnification of both cancer (B) and dysplasia (C). ×250. D, cyclin A immunoreactivity was restricted to the cells of the basal layer in the transitional epithelium of the urinary tract. ×250.

**Table 1** Summary of the relationship between the cyclin A immunoreactivity and clinicopathological factors

<table>
<thead>
<tr>
<th>Cyclin A− (91 cases)</th>
<th>Cyclin A+ (29 cases)</th>
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<tbody>
<tr>
<td>Age</td>
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<td>&lt;70</td>
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<td>70-80</td>
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<td>1: 1 &gt; 2</td>
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<td>10</td>
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<tr>
<td>Growth</td>
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<td>Invasive</td>
<td>70</td>
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<td>PIT</td>
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<td>Chemo.</td>
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<td>(−)</td>
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Subjects followed by the method of the Japanese Urological Association and the Society of Pathology (29). Grade, tumor grade; pT, depth of penetration; ly or v, lymphatic or venous invasion; m or n, distant organ or lymph node metastasis; growth, tumor growth pattern; NNT, nonpapillary noninvasive type; PNT, papillary noninvasive type; NIT, nonpapillary invasive type; PIT, papillary invasive type.

Statistical Analysis. Table 1 shows the relationships between the rates of detection of overexpressed cyclin A in primary tumors and clinical or pathological features. The relationships of the cyclin A-positive cases with high nuclear grade (P < 0.01) and also with an invasive tumor growth pattern (P < 0.05) were both statistically significant. In contrast, no significant correlation was detected between cyclin A overexpression and the other clinical and pathological parameters, such as sex, age, depth of penetration, lymphatic or venous invasion, distant organ or lymph node metastasis, and treatment with chemotherapy.

**Association between Cyclin A Expression and Prognosis.** Fig. 3 shows the Kaplan-Meier survival curves based on a comparison of the two groups; one group consists of the patients with positive immunostaining with cyclin A and the other with negative immunostaining with cyclin A. In all 117 patients tested, the postoperative 10-year survival rate of the cyclin A-positive group (n = 28) was 26.5%, whereas that of the cyclin A-negative group (n = 89) was 62.4%. There was a significant difference between these two groups (P < 0.01; Fig. 3A). Of the 72 patients with low grade TCC (grade 2 > 1; 3 < 2, or 1), the postoperative 9-year survival rate of the cyclin A-positive group (n = 11) was 35.4%, whereas that of the cyclin A-negative group (n = 61) was 70.2%; the difference between these rates was statistically significant (P < 0.05; Fig. 3B). Of the 45 patients with high grade TCC (grade 3 > 2, or 1), the postop-
Fig. 3 The cumulative Kaplan-Meier survival curves of patients with carcinoma of renal pelvis and ureter. There are statistically significant differences between the cyclin A-positive and cyclin A-negative group in all 117 patients tested (A, \( P < 0.01 \)) and in patients with low grade TCC (B, \( P < 0.05 \)) and high grade TCC (C, \( P < 0.05 \)). Although no significant difference in the survival rate for the patients with noninvasive type of TCC is detected (D), there is a statistically significant difference between the cyclin A-positive and cyclin A-negative group for the patients with the invasive type of TCC (E, \( P < 0.05 \)).

The postoperative 9-year survival rate of the cyclin A-positive group (\( n = 17 \)) was 13.8\%, whereas that of the cyclin A-negative group (\( n = 28 \)) was 48.2\%; the difference between these rates was also statistically significant (\( P < 0.05 \); Fig. 3C). In the tumor growth pattern, there was no significant difference in the survival rate for the 25 patients with the noninvasive type of TCC between cyclin A-positive (\( n = 4 \)) and cyclin A-negative groups (\( n = 21 \); Fig. 3D). In contrast, the postoperative 10-year survival rates for the 92 patients with the invasive type of TCC was 18.6\% for the cyclin A-positive group (\( n = 24 \)) and 50.3\% for the cyclin A-negative group (\( n = 68 \)), respectively. There was a significant difference between these groups (\( P < 0.05 \); Fig. 3E).
To determine the most informative combination of independent factors for prognosis, the variables identified as statistically significant in predicting survival (tumor grading, depth of penetration, lymphatic or venous invasion, distant organ or lymph node metastasis, tumor growth pattern, and cyclin A overexpression) were subjected to a multivariate analysis using Cox’s stepwise proportional hazard model. Each of these factors had a statistically significant effect on the survival rate. A stepwise selection of these factors was made, based on the relative magnitude of their contribution to survival. This analysis demonstrated that the most important factor affecting survival was the distant organ and/or lymph node metastasis (P < 0.001). Moreover, cyclin A overexpression was also an important factor affecting survival (P < 0.01) as well as tumor grading (P < 0.05) and the tumor growth pattern (P < 0.05).

DISCUSSION

In this study, we directly examined the relevance of cyclin derangement to in vivo conditions by immunohistochemically measuring and comparing the expression of cyclin A protein in tumor samples from patients with TCC. Immunohistochemistry using antibodies to cyclin A is optimally designed for precise measurement of the expression rates of this protein and its pattern of expression in an individual tumor cell, and this technique may be suitable for screening. In the present study, 24.2% (29 of 120) of the tumor samples had increased levels of cyclin A protein predominantly detected in the nuclei of cancer cells. Recently, Keyomarsi and Pardee (6) also showed the unscheduled patterns and timing of overexpression of cyclin A through G$_1$ to G$_2$-M phase in human breast cancer cell lines (6). Our present data and their findings suggest that, in the synchronized populations of cyclin A-positive tumor cells observed, cyclin A protein is no longer a cell cycle regulator; instead, it appears to be constitutively regulated in the cell cycle. However, in these cyclin A-positive tumors, mitotic figures were commonly observed, and the intensity of positive nuclear staining varied between tumor cells within the identical tumor. These observations support the hypothesis that cyclin A protein is not constantly expressed and may be degraded or expelled from the nucleus during progression of the cell cycle.

Urothelial TCC usually exhibits significantly different behavior, depending on the nuclear grade of the cancer cells and the patterns of growth. The present study showed that tumors with cyclin A protein overexpression became progressively more abundant with an increase in the degree of tumor grade (P < 0.01) and tumor invasion (P < 0.05). Thus, our results suggest that cyclin A overexpression may be associated with the clinical behavior of high grade and progressive TCCs. In the present study, our findings also indicated that the groups with a high level of cyclin A expression was associated with a significantly poorer prognosis than the groups without its expression. These in vivo findings suggest that cyclin A overexpression is also related to the poor prognosis for the patients with renal pelvic and ureteral TCCs.

We also investigated the expression of cyclin A in samples containing normal and dysplastic lesions adjacent to the primary tumors. One important finding in this study was that approximately 58% (17 of 29) of the dysplastic lesions adjacent to cyclin A-positive tumors eventually featured cyclin A overexpression. It is also of interest to note that physiological levels of cyclin A protein was detected in the subsets of cells of the basal layer in the nonneoplastic transitional epithelium. This observation supports the importance of abnormal levels of expression of this protein in urothelial dysplasia. In the present study, these findings suggest that unscheduled overexpression of cyclin A may be due to loss of the cell cycle regulation at an early stage of the pathogenesis of some cases of urinary neoplasms. In addition, detection of cyclin A overexpression in premalignant lesions of the urinary tract may potentially be useful in diagnosis and determination of their roles in the natural history of TCC.

Although the molecular basis for the positive immunostaining is still indefinite, this is the first report of frequent cyclin A protein overexpression in a large series of primary human TCCs including dysplasia. In addition, the present study also demonstrated that immunohistochemical detection of cyclin A protein is useful in the search for novel and potentially useful prognostic markers in TCC also. Recent reports have further demonstrated the presence of a link between oncogenesis and the cell cycle by correlating the deranged expression of cyclins to the loss of growth control (4, 6, 14). Therefore, the frequent alterations of cyclin A protein in tumor cells are also suggestive of its role as an oncogene. Up to the present time, most investigations into the relationship between the expression of cyclin and carcinogenesis were mainly carried out in vitro using certain cell lines. Further comprehensive studies using excised human carcinoma tissue samples from greater numbers of human tumors and including measurement of DNA and/or RNA levels will be needed to elucidate the function and clinical significance of cyclin A overexpression.

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REFERENCES


Cyclin A overexpression in carcinoma of the renal pelvis and ureter including dysplasia: immunohistochemical findings in relation to prognosis.

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