Expression of Cyclin D1, but not Cyclins E and A, Is Related to Progression in Bilharzial Bladder Cancer

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ABSTRACT

The present study was conducted to analyze the alterations affecting cyclins D1, E, and A in bilharzial bladder cancer and to assess their potential clinical significance. A total of 125 cases were examined. Histopathological subtypes included 68 squamous cell carcinomas, 55 transitional cell carcinomas, and 2 adenocarcinomas. Immunohistochemical analyses were performed using a panel of well-characterized antibodies. The results were correlated with proliferative analyses were performed using a panel of well-characterized antibodies.

The cyclin D1-positive phenotype, defined as the identification of positive immunoreactivity in the nuclei of >20% of tumor cells, was found in 33 of 107 (31%) evaluable cases. A significant association was observed between the cyclin D1-positive phenotype and deep muscle invasion (P = 0.02), high tumor grade (P = 0.02), and Ki67 high proliferative index (P = 0.03). The cyclin E-positive phenotype, defined as per cyclin D1, was found in 79 of 106 (75%) evaluable cases. The cyclin A-positive phenotype, defined using the above criteria, was identified in 60 of 108 (56%) evaluable cases. No statistically significant association was found between cyclins E or A and clinicopathological parameters or proliferative index. However, there was a strong association between the expression of cyclin D1 and the coexpression of cyclins A and/or E (P = 0.05). Ki67 proliferative index was considered high when >20% of tumor cells displayed positive nuclear staining, a phenotype that was observed in 99 of 115 (86%) cases. These data support the hypothesis that cyclin D1 activation determines the evolution of a particular subset of aggressive bladder tumors. In addition, cyclins E and A seem to follow an unscheduled pattern of expression, based on the high frequency of identifying a positive phenotype for these cyclins and the lack of correlation between their expression and Ki67 high proliferative index. It may be postulated that the expression of G1 cyclins is deregulated in bilharzial bladder cancer, and that cyclin D1 acts as an oncogenic event in these neoplasms. Moreover, the moderate number of tumors displaying the cyclin D1-positive phenotype (31%) versus the high frequency observed for both cyclins E (75%) and A (56%), suggests a short G1 disbalanced by a long S phase and a rapid transversal of the cell cycle, evidenced by a high Ki67 index observed in 86% of these cases. This imbalance in the cell cycle, together with alterations reported on the p53 pathway, might underline the accumulation of DNA damage and the aggressive clinical course of bilharzial bladder cancer.

INTRODUCTION

BBC has been reported to be a neoplastic disease of slow growth rate with a tendency to recur locally rather than to metastasize (1–3). However, a recent report from our group showed that 86% of the BBCs analyzed had a high proliferative index and a low rate of apoptosis (4). An overexpression of the protein encoded by the MDM2 gene was also found in 60% of the cases studied, which may account for the frequent inactivation of the p53 control pathway observed in BBC (4). The discrepancy between the clinical impression that BBC is a slowly growing neoplasm and our findings regarding alteration in the p53 pathway prompted us to undertake an in-depth analysis of cell cycle-regulatory subunits concentrating on the G1 cyclins.

Cell cycle transitions are controlled by functional heterodimers composed of a cyclin acting as a regulatory subunit and a cyclin-dependent kinase, which acts as the catalytic component (5). In the present study, we have examined the expression of cyclins D1, E, and A in a cohort of 125 well-characterized BBC cases to further characterize the deregulation of G1 observed in BBC. Multiple cyclins have been isolated and characterized, and a temporal map of their expression has been delineated (5). It is postulated that the complexes formed by cyclin D1 and cdk4 govern G1 progression, whereas cyclin E-cdk2 controls entry into S phase. Cyclin A-cdk2 affects the regulation through the S phase (5). Several studies suggest that gene amplification and overexpression of cyclin D1 and cdk4 are oncogenic events in certain tumors (6, 7). However, alterations affecting cyclins E and A seem to be less frequent. Cyclin

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3 The abbreviation used is: BBC, bilharzial bladder cancer.
E has been shown to be amplified in only a few tumor cell lines, whereas cyclin A has been found to be altered in a single report dealing with hepatocellular carcinoma (8). Nonetheless, sustained overexpression of cyclin E has been observed in many neoplasms including colon, stomach, and endometrial carcinomas (8). In breast cancer, several groups have demonstrated the negative impact of disregulated cyclins on prognosis (9–12). The expression patterns of the cyclin genes analyzed in the present study were selected on the basis of their role in the regulation of the G1, as well as the G1-S-phase transition. The alterations identified have been further correlated with the proliferative index as well as relevant clinicopathological parameters in an attempt to define their potential biological significance in BBC.

PATIENTS AND METHODS

Patients. A cohort of 125 patients with bilharzial-related bladder tumors (BBC) was evaluated. Schistosomiasis infection was confirmed by the presence of ova on histological sections in all cases. Demographic data on this group could be summarized as follows: 99 patients were males, and 26 were females; the mean age was 53 years. Tissues were obtained from the Pathology Department of the National Cancer Institute of Cairo in Egypt. Samples were formalin-fixed paraffin-embedded tissue specimens. Representative H&E-stained sections were examined to evaluate the pathological type, grade, and stage of the tumors to be analyzed. Sixty-eight cases were squamous cell carcinomas, 55 cases were transitional cell carcinomas, and 2 cases were adenocarcinomas. Tumors were staged as 3 P1, 4 P2, 39 P3a, 70 P3b, 7 P4a, and 2 P4b. Fifteen tumors were classified as low grade (grade 1), 79 tumors were classified as intermediate grade (grade 2), and 31 tumors were classified as high grade (grade 3) lesions. Twenty-five patients (20% of cases) had lymph node infiltration on pathological examination. In addition, for 18 cases, biopsies from areas with normal urothelial mucosa (n = 12) or squamous metaplasia (n = 6) were analyzed as internal normal controls in the study. All patients were subjected to radical cystectomy, with the exception of one case in which simple cystectomy was performed. No preoperative radiation therapy or chemotherapy was administered to any of these patients.

Monoclonal Antibodies and Immunohistochemistry.

The following well-characterized antibodies and corresponding final working dilutions were used for the present study: anti-cyclin D1 mouse monoclonal antibody (Ab-3; clone DCS-6; IgG1; Oncogene Calbiochem), 1:500 dilution; anti-cyclin A mouse monoclonal antibody (clone BF-683; IgG1; PharMin- gen), 1:20 dilution; and anti-cyclin E rabbit purified antiserum, 1:800 dilution.4 MlgS-Kpl, a mouse monoclonal antibody of the same subclass as the primary antibodies listed above, and a rabbit preimmune serum were used as a negative controls at similar working dilutions. Sections were immersed in boiling 0.01% citric acid (pH 6.0) for 15 min under microwave treatment to enhance antigen retrieval, allowed to cool, and incubated with primary antibodies or antiserum overnight at 4°C. Biotinylated horse antimouse IgG and goat antirabbit immunoglobulin antibodies were used as secondary reagents applied for an incubation period of 1 h (Vector Laboratories, Burlingame, CA; 1:500 dilution), followed by avidin-biotin peroxidase complexes incubated for 30 min (Vector Laboratories; 1:25 dilution; Ref. 4). Diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. Nuclear immunoreactivities were classified into two categories defined as follows: negative, <20% of tumor cells displaying nuclear immunostaining; and positive, ≥20% of tumor cells with nuclear immunostaining.

Statistical Methods. For clinicopathological variables, we classified patients into three groups according to their histological types. These included squamous cell carcinoma, transitional cell carcinoma, and adenocarcinoma. Pathological stage was grouped into two subgroups: ≤P3a and ≥P3b (deep muscle invasion). Grade was categorized into two groups: grade 1 and grades 2 and 3. Lymph node metastases were recorded as negative or positive. For biomarker variables, we used a cut-point of 20% (<20% as negative and ≥20% as positive) for the three immunophenotypic determinants including expression of cyclins D1, A, and E as well as to evaluate high Ki67 proliferative index (4).

Data analyses were conducted to assess: (a) the correlations among immunophenotypic variables; (b) the relationship between immunophenotypic variables and clinicopathological variables such as stage, grade, histology type, and lymph node status; (c) the association between coexpression of immunophenotypic variables and clinicopathological variables. Fisher’s exact test (13) was used to assess these associations, and two-tailed P values were used as a significant level. For variables with more than two categories, the dose-response relationship was assessed by the trend test using the Mantel-Haenszel method (14). The FREQ procedure in SAS was used for this study (15).

RESULTS AND DISCUSSION

Table 1 summarizes laboratory data in relation to clinicopathological parameters, including histological subtype, tumor stage, tumor grade, lymph node status, and Ki67 proliferative index. Fig. 1 illustrates the immunohistochemical staining patterns of cyclins D1, A, and E in representative cases as well as in squamous metaplasia.

The cyclin D1-positive phenotype was observed in 33 of 107 evaluable cases (31%; Fig. 1, A and B). Cyclin D1-positive cases were more commonly identified among patients with higher tumor grade (P = 0.02), advanced-stage tumors (P = 0.02), and high Ki67 proliferative index (P = 0.03). In addition, a trend was observed between the cyclin D1-positive phenotype and transitional histology (P = 0.08). There was no correlation between cyclin D1 expression and lymph node status. However, the analysis of the association between cyclin D1 overexpression and advanced stage in lymph node-negative patients showed that it remained statistically significant (P = 0.03). In addition, there was a significant association between the expression of cyclin D1 and the coexpression of cyclin E and/or cyclin A (P = 0.05). We also observed a heterogenous pattern of nuclear staining in 3 of 6 mucosa showing squamous metapla-
Bladder tumors with deep muscle invasion (P phenotype and the evolution of a particular subset of aggressive proliferative index (P expression of cyclin D1 was strongly associated with high Ki67 previously reported data on other tumor types, including carcinoma of the breast and head and neck cancer (6, 7). The cyclin D1-positive phenotype in 31% of conventional transitional cell carcinoma. Combined data from these studies show that 29 cases (14%) had amplification of this region; however, these reports lack correlative analysis of laboratory data and clinicopathological parameters (18-21). The 31% incidence of the cyclin D1-positive phenotype observed in this study cannot be compared with the previous figure, because expression data were not reported in the above-mentioned studies.

The relatively high frequency of the positive phenotype for cyclins E (75%) and A (56%) in BBC and the lack of their expression data were not reported in the above-mentioned studies. The cyclin A-positive phenotype was observed in 60 of 108 evaluable cases (60% of the total); however, all 12 normal urothelial samples analyzed were nonreactive.

The cyclin E-positive phenotype was detected in 79 of 106 evaluable cases (75%; Fig. 1D). A significant association was found between the cyclin E-positive phenotype and transitional histology subtype (P = 0.01). However, there was no association between cyclin E and the other clinicopathological variables analyzed. We also observed a heterogenous pattern of nuclear staining in all squamous metaplasia mucosa studied (n = 6; Fig. 1C); however, all 12 normal urothelial samples analyzed were nonreactive (data not shown).

The cyclin A-positive phenotype was observed in 60 of 108 evaluable cases (56%; Fig. 1E). All normal urothelium or squamous metaplasia samples analyzed had undetectable levels of cyclin A (data not shown). In addition, there was no correlation between the cyclin A-positive phenotype and histology subtype, tumor grade, tumor stage, lymph node status, or Ki67 proliferative index.

The frequency of cyclin D1-positive phenotype in 31% of evaluable BBC cases reported in this study is comparable to previously reported data on other tumor types, including carcinoma of the breast and head and neck cancer (6, 7). The expression of cyclin D1 was strongly associated with high Ki67 proliferative index (P = 0.03), which, together with the significant association with the coexpression of cyclins E and/or A (P = 0.05), indicates a potential correlation between a short G1 in the context of a rather rapid traversal of the cell cycle. Moreover, the strong association between the cyclin D1-positive phenotype and the evolution of a particular subset of aggressive bladder tumors with deep muscle invasion (P = 0.02) and high grade (P = 0.02) relates cyclin D1 to tumor progression in BBC. These findings are in agreement with data from previous studies reporting that cyclin D1 overexpression, with or without gene amplification, was a prognostic determinant of poor disease outcome (16). Cyclin D1 gene amplification occurs consistently in certain neoplasms as part of the 11q13 amplicon region. This situation has been described in esophageal carcinoma as well as in head and neck cancer (17). However, in breast cancer, a discrepancy between cyclin D1 gene amplification (13% of cases) and cyclin D1 overexpression (50%) was observed, suggesting that protein overexpression, but not necessarily gene amplification, is associated with poor prognosis (17).

In bladder cancer, four studies dealing with potential amplification of cyclin D1 as assessed by genetic analysis of the 11q13 region have been reported, studying a total of 206 cases of conventional transitional cell carcinoma. Combined data from these studies show that 29 cases (14%) had amplification of this region; however, these reports lack correlative analysis of laboratory data and clinicopathological parameters (18-21). The Table I Summary of data in relation to immunophenotype profile

<table>
<thead>
<tr>
<th>Character</th>
<th>Cyclin D1</th>
<th>Cyclin E</th>
<th>Cyclin A</th>
</tr>
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<tr>
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<td>40/61</td>
<td>35/60</td>
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<tr>
<td>Transitional</td>
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<td>39/44</td>
<td>25/46</td>
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<tr>
<td>Adeno</td>
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<td>0/2</td>
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<td>0.01</td>
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<td>24/36</td>
<td>22/41</td>
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<tr>
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<tr>
<td>P</td>
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<td>2</td>
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<tr>
<td>P</td>
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<td>0.3</td>
<td>0.1</td>
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<tr>
<td>Lymph node</td>
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</tr>
<tr>
<td>Node-</td>
<td>24/85</td>
<td>62/85</td>
<td>45/86</td>
</tr>
<tr>
<td>Node+</td>
<td>8/21</td>
<td>16/20</td>
<td>15/21</td>
</tr>
<tr>
<td>P</td>
<td>0.4</td>
<td>0.1</td>
<td>0.5</td>
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<tr>
<td>Ki67 proliferative index</td>
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<td></td>
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<tr>
<td>&lt;20</td>
<td>1/14</td>
<td>9/16</td>
<td>4/13</td>
</tr>
<tr>
<td>≥20</td>
<td>31/87</td>
<td>66/85</td>
<td>53/89</td>
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<tr>
<td>P</td>
<td>0.03</td>
<td>0.1</td>
<td>0.07</td>
</tr>
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</table>

No. a, number of positive cases.

Percentage of positive cases (≥20% tumor cells immunoreactive).

Ki67 proliferative index was considered high when ≥20% tumor cells displayed a positive MIB1 nuclear staining.

Squamous 13/58 22 40/61 65 35/60 58
Transitional 18/47 38 39/44 88 25/46 54
Adeno 2/2 100 0/1 0 0/2 0
Stage P (squam. vs. trans.) 0.08 0.01 0.6
Stage <p3a 6/38 16 24/36 66 22/41 53
Stage p3b <p3c 27/69 39 55/70 78 38/67 56
P 0.02 0.2 0.8
Grade 1 0/12 0 8/13 61 5/14 35
2 33/94 35 70/92 76 55/93 60
P 0.02 0.3 0.1
Lymph node Node- 24/85 28 62/85 73 45/86 52
Node+ 8/21 38 16/20 80 15/21 71
P 0.4 0.1 0.5
Ki67 proliferative index <20 1/14 7 9/16 56 4/13 30
≥20 31/87 36 66/85 77 53/89 55
P 0.03 0.1 0.07
Expression of Cyclin D1 Is Related to BBC Progression

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endothelial cells are to cyclin D1, cyclin A, and cyclin E. A Photomicrographs of selected cases of bilharzial-related bladder cancer (BBC) studied using immunohistochemical staining with antibodies Fig. 1 expression of both cyclins D1 and E in shortening the G1 D, with squamous metaplasia. G1 and an increase in the length of the S phase + shortened G1, and an increase in the length of the S phase + G2 + M phase. In addition, the additive effect of the ectopic expression of both cyclins D1 and E in shortening the G1 interval relative to the expression of any single cyclin has been also reported (24). Thus, there is a good correlation in rodent and human experimental systems, primary tumor data, and BBC. However, the mechanism by which unscheduled G1 cyclin expression may lead to prolongation of the remainder of the cycle is not clear. Regardless, the imbalance in cell cycle control and the high incidence of p53 alterations reported in BBC (4) might explain why DNA damage accumulates in these cells. It may be speculated that unscheduled cyclin activity may affect the fidelity of the replication apparatus and that the prolonged S phase may allow an increase in DNA damage, which would be

Fig. 1 Photomicrographs of selected cases of bilharzial-related bladder cancer (BBC) studied using immunohistochemical staining with antibodies to cyclin D1, cyclin A, and cyclin E. A and B, intense nuclear staining of clone DCS-6 (Ab-3) to cyclin D1 in tumor cells. Note that fibroblasts and endothelial cells are negative for this antibody. C, heterogeneous nuclear immunoreactivities of anti-cyclin E antiserum in a bladder mucosa affected with squamous metaplasia. D, a heterogeneous pattern of staining of anti-cyclin E antiserum in an invasive BBC. E, an invasive BBC displaying a strong reactivity to clone BF-683 to cyclin A. Note that the mitotic cells identified are nonreactive for anti-cyclin A antibodies. Original magnifications: A and D, ×200; B, C, and E, ×400.

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favored by the absence of the genomic integrity checkpoints mediated by p53. In this regard, similar circumstances characterize Bloom's syndrome, marked by a hypermutation phenotype produced by alterations of the encoded gene product, which shares homology with helicases specifically involved in replicative control (25).

The postulate that deregulated expression of cyclin D1 in early G1 may drive the cell to a point where it gets more susceptible to additional DNA damage was previously studied in vitro (26). Using rat embryonic fibroblast, cotransfection of ras and mutant p53 transformants with the cyclin D1 expression plasmid resulted in reduced serum dependency in vitro. Furthermore, the transformants expressing exogenous cyclin D1 grew faster when injected into nude mice (26). These results indicate that deregulated expression of cyclin D1 in early G1 may reduce the requirement for growth factors and may produce in vivo growth.

In summary, data from the present study support the hypothesis that cyclin D1 acts as an activated oncogenic event in BBC and that it may determine the evolution of a particular subset of aggressive bladder tumors. This is supported by the significant association between the cyclin D1-positive phenotype with deep muscle invasion and high tumor grade. The prominent number of cases displaying the cyclin E and cyclin A-positive phenotype and the lack of correlation between these phenotypic patterns and high Ki67 proliferative index support the postulate of their unscheduled expression. The disbalance produced by these events may generate a relatively short G1 followed by a prolonged S phase in the context of a rapid traversal of the cell cycle.

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Expression of cyclin D1, but not cyclins E and A, is related to progression in bilharzial bladder cancer.

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