Alterations Affecting the p53 Control Pathway in Bilharzial-related Bladder Cancer

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ABSTRACT

Bilharzial-related bladder carcinoma (BBC) is the most common malignant neoplasm in Egypt, also occurring with a high incidence in other regions of the Middle East and East Africa. The clinical and pathological features of BBC are different than those described for the conventional transitional cell carcinoma of the bladder, including the high incidence of squamous cell carcinoma reported in BBC and the fact that over 90% of BBC cases at presentation are advanced-stage tumors (P3 and P4). This study was conducted to better define the phenotypic alterations associated with BBC, affecting the p53 cell cycle control pathway, including altered patterns of expression of downstream effector proteins such as mdm2 and p21/WAF1. A well-characterized cohort of 125 patients affected with bilharzial-related bladder tumors was studied. Tumors were classified as squamous carcinomas (n = 68), transitional cell carcinomas (n = 55), or adenocarcinomas (n = 2). The products encoded by TP53, mdm2, and p21/WAF1 genes were analyzed by immunohistochemistry. Furthermore, the patterns of expression of these molecules were correlated with the Ki67 proliferative index. In addition, the microanatomical distribution of programmed cell death was assessed in a subset of tumors, using the so-called terminal deoxynucleotidyl transferase-mediated nick end labeling method. p53 nuclear overexpression was identified in 25 (20%) of 125 cases. Nuclear overexpression of mdm2 was detected in 74 (59.2%) of 125 cases. There was a statistically significant association between coexpression of both p53 and mdm2 and detection of lymph node metastases (P = 0.04). p21/WAF1 expression was detected in 87 (72%) of 121 evaluable cases. A high Ki67 proliferative index was observed in 99 (86%) of 115 evaluable cases. There was a statistically significant association between high Ki67 proliferative index and mdm2-positive phenotype (P = 0.005) and deep muscle invasion (P3b; P = 0.026) as well as lymph node metastases (P = 0.039). Apoptosis was observed in terminally differentiated tumor cells identified in the superficial layers of well-differentiated squamous carcinoma or exfoliating cells in transitional lesions. However, only rare apoptotic tumor cells were found in basal or suprabasal layers as well as in the invasive elements of the neoplasms studied. These results suggest that the frequency of p53 nuclear overexpression in BBC is lower than that reported for conventional transitional cell carcinoma. Nevertheless, tumors with p53 alterations have a greater propensity to progress. The prominent number of cases displaying an mdm2-positive phenotype suggests that this may be an early incident in BBC and should be regarded as a potential oncogenic phenomenon. This is supported by the significant correlation between high Ki67 proliferative index and mdm2 overexpression. The association of an aggressive clinical course with the coexpression of both p53 and mdm2 products might be viewed as a cooperative effect that develops in tumor progression.

INTRODUCTION

BBC is the most common malignant neoplasm in Egypt, accounting for more than 25% of all diagnosed cancer cases (1). These tumors also occur with a high incidence in other regions of the Middle East and East Africa (2, 3). The clinical and pathological features of BBC are different than those described for the cTCC of the bladder. Clinically, the mean age at diagnosis is two decades younger in BBC than in cTCC. Pathologically, over 90% of BBCs present with invasive disease (P3 or P4) as opposed to superficial disease (Pa, Pis, or P1) in over 70% of cTCC (4). The most striking difference is the high incidence in BBC of SSC, ranging from 44–80% (5), whereas <7% of bladder tumors developing in Western countries have a squamous histology (6). Finally, BBC with SCC (BBC-SSC) features tend to recur locally and rarely metastasize. Aggressive treatment strategies have been adopted, mainly radical cystectomy with or without radiation therapy, in an attempt to achieve local control of the disease. Nevertheless, 5-year survival rates are poor and hardly exceed 40% (4).

Detailed molecular genetic studies of cTCC have led to a working hypothesis of tumorigenesis and progression (7–9). Similar studies of BBC are limited. In a study of 21 BBC-SSC cases, point mutations of H-ras were observed in 3 cases (16%:...
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Ref. 10). Data on detection of TP53 mutations is conflicting, ranging from 35% (2, 10) to 60% (11) and 86% (12) of cases studied. More recently, deletions affecting p16INK4A and/or other microsatellites on the short arm of chromosome 9 were found in 11 of 12 cases of BBC-SCC (11).

A limitation of these studies is that they focused on a single marker in a small number of cases. We have undertaken a collaborative effort aimed at the molecular characterization of BBC in a large and well-characterized cohort of patients. The present study reports alterations affecting cell cycle regulators involved in the p53 control pathway in 125 patients affected with BBC. These alterations have been further correlated with proliferative index and apoptosis, as well as relevant clinicopathological parameters, in an attempt to define their biological significance in BBC.

MATERIALS AND METHODS

Patients. A cohort of 125 patients with bilharzial-related bladder tumors (BBC) were evaluated. Schistosomiasis infection was confirmed by the presence of ova on histological sections in all cases. Demographic data on this group may be summarized as follows: 99 patients were males, and 26 were females; and the median age was 53 years (range, 31–72 years). Tissues were obtained from the Pathology Department at 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 SCCs, 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 lesions (grade 1), 78 tumors were classified as intermediate-grade lesions (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, and all of them staged as >P3b. In addition, 15 cases had adjacent normal mucosa displaying normal urothelium (n = 6) or squamous metaplasia (n = 9). These tissues were also analyzed as internal normal controls in the study. All patients were subjected to radical cystectomy, with the exception of one case in which a simple cystectomy was administered to any of these patients.

Monoclonal Antibodies and Immunohistochemistry. The following well-characterized mouse monoclonal antibodies and corresponding final working dilutions were used for the present study: anti-p53 monoclonal antibody PAB1801 (Ab-2; Oncogene Science; 1:500 dilution); anti-mdm2 monoclonal antibody 2A10 (a gift from Dr. Arnold Levine, Princeton University, Princeton, NJ; 1:500 dilution); anti-p21 monoclonal antibody WAF1 (Ab-1; Oncogene Science; 1:20 dilution); and anti-Ki67 monoclonal antibody MIB1 (Immunotech; 1:50 dilution). MlgS-Kpl, a mouse monoclonal antibody of the same subclass as the primary antibodies listed above, was used as a negative control at similar working dilutions. Sections were subsequently immersed in boiling 0.01% citric acid (pH 6.0) for 15 min to enhance antigen retrieval, allowed to cool, and incubated with primary antibodies overnight at 4°C. Biotinylated horse anti-mouse IgG antibodies were applied for 1 h (Vector Laboratories, Burlingame, CA; 1:500 dilution), followed by avidin-biotin peroxidase complexes for 30 min (Vector Laboratories; 1:25 dilution). Diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. Nuclear immunoreactivities were classified into two categories: negative (<20% of tumor cells displaying nuclear staining) and positive (≥20% of tumor cells with nuclear staining; Refs. 13 and 14). Ki67 proliferative index was considered high when ≥20% of tumor cells displayed a positive MIB1 nuclear staining pattern.

TUNEL Assay for the Detection of Apoptotic Cells. In a subset of selected cases, TUNEL was used to study the microanatomical distribution of programmed cell death. This assay was performed using a modification (15) of the method originally described by Gavril et al. (16). The method is based on the specific binding of terminal deoxynucleotidyl transferase to the 3'-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide. Briefly, after the exposure of nuclear DNA of histological sections by proteolytic treatment, terminal deoxynucleotidyl transferase was used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The signal was amplified by avidin-biotin peroxidase complexes and visualized by diaminobenzidine deposition, enabling conventional histochecmical identification of reactive nuclei by light microscopy.

Statistical Analysis. For clinicopathological variables, we classified patients into three groups according to their histological types. These included SCC, transitional cell carcinoma, and adenocarcinoma. Pathological stage was grouped into two subgroups, ≤P3a and ≥P3b. Grade was categorized as into two groups, grade 1 and grades 2 and 3. Lymph node metastasis was recorded as negative and positive. For biomarker variables, we used a cut-point of 20% (<20% as negative and ≥20% as positive) for 4 immunophenotypic variables, including patterns of p53, mdm2, p21/WAF1, and Ki67 in the data analysis, to avoid potential false positive error.

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; and (c) the association between coexpression of immunophenotypic variables and clinicopathological variables. Two-tailed Fisher’s exact test (17) 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 trend test using the Mantel-Haenszel method (18). The FREQ procedure in SAS was used for this study (19).

RESULTS

Table 1 summarizes data in relation to clinicopathological parameters, including tumor subtype, tumor stage, tumor grade, lymph node status, and immunophenotype profiles. Fig. 1 illustrates the immunohistochemical staining patterns of p53, mdm2, p21/WAF1, and MIB1 in representative cases. Fig. 2 depicts the microanatomical localization of apoptotic signals identified by the TUNEL method in relation to the proliferative activity in a selected case.
Nuclear overexpression of p53 was observed in 25 of 125 cases (20%; Fig. 1A). However, there were no cases of normal urothelium or squamous metaplasia showing p53 immunoreactivities. There was no difference between p53-positive phenotype and distinct pathological subtypes of the tumors analyzed. Nevertheless, p53-positive cases were more commonly identified among patients with higher-grade and advanced-stage tumors. However, the difference was not statistically significant.

Nuclear overexpression of mdm2 was observed in 74 of 125 cases (59%; Fig. 1B). We also observed a heterogeneous pattern of nuclear staining in two of nine mucosa showing squamous metaplasia; however, all six normal urothelial samples were unreactive. Intense nuclear staining (>70% tumor cells with positive nuclei) was identified in 31 cases (25%). A heterogeneous nuclear staining pattern was observed in fibroblasts in some cases. There were no differences between mdm2-positive phenotype and pathological subtype, tumor grade, tumor stage, or lymph node status. However, a statistically significant association was found between coexpression of p53 and mdm2 (n = 17 cases) and development of lymph node metastases (P = 0.04).

Nuclear overexpression of p21/WAF1 was detected in 87 of 121 evaluable cases (72%; Fig. 1C). Fifteen of the 25 cases that showed p53 nuclear overexpression displayed coexpression of p21 in the nuclei of tumor cells. We did not observe p21 immunoreactivities in normal urothelium or mesenchymal elements, including fibroblasts. There was no statistically significant association between p21-positive phenotype and the clinicopathological variables analyzed.

A high Ki67 proliferative index was observed in 99 of 115 evaluable cases (86%). Neoplastic cells located in the basal and suprabasal layers of tumors with squamous differentiation displayed frequent Ki67-positive nuclei (Fig. 2A). Intense nuclear staining in over 70% of tumor cells was observed in 38 of 99 positive cases (38%; Fig. 1D). Statistically significant correlations between MIB1-positive phenotype and deep muscle invasion (P = 0.026) as well as metastatic lymph node status (P = 0.039) were observed. In addition, high Ki67 proliferative index was found to be correlated with mdm2 overexpression (P = 0.005).

Positive nuclear staining by TUNEL assay, representing microanatomical identification of cells undergoing programmed cell death, was mainly observed in terminally differentiated tumor cells identified in the luminal layer(s) of well-differentiated squamous carcinomas (Fig. 2B) and in exfoliating cells of transitional BBC (data not shown). However, only rare apoptotic cells were found in the basal or suprabasal layers of squamous carcinomas as well as in the invading tumor cells of the neoplasms studied (data not shown).

### DISCUSSION

Several studies have revealed that TP53 mutations and altered patterns of p53 expression are common events in cTCC and are associated with an aggressive tumor behavior (13, 20–23). In this study we are reporting an overall frequency of 20% for p53 nuclear overexpression in BBC, which is lower than that reported for cTCC (13, 14, 20–23). We observed a higher frequency of p53 nuclear overexpression in patients with deep muscle invasion (P3b), tumors displaying intermediate and high grade, and cases with metastatic lymph node status. However, differences did not reach statistical significance, probably due to the relatively small number of cases that showed a p53-positive phenotype. Results from this study suggest that p53 alterations are common but not frequent events in BBC.

Interpreting the results of molecular analyses of TP53 and altered p53 expression has been limited by the utilization of dissimilar methodologies on a variety of different epidemiological backgrounds (10–12). Another consideration is that most of the studies have focused on comparing only the squamous subtype of BBC and cTCC, underestimating the fact that BBC presents as both transitional and squamous carcinoma. Finally,
there are important concerns regarding the quality of high molecular weight DNA extracted from BBC, because these tumors usually have extensive areas of necrosis. As a matter of fact, we attempted DNA extraction in a subset of 32 cases from the present study for which frozen material was available. We found that in the majority of the cases, the DNA extracted was suboptimal for molecular studies, preventing an in-depth analysis of TP53 and mdm2 mutations (data not shown).

We further analyzed the role of p53 alterations in BBC by studying the expression pattern of two of the genes under its transcriptional regulation, namely mdm2 and p21/WAF1. The mdm2 gene maps to the long arm of chromosome 12 and encodes a 90-kDa zinc-finger protein (mdm2), which contains a p53-binding site (24, 25). It has been shown that mdm2 proteins bind to p53 and act as negative regulators, inhibiting p53 transcriptional activity (24–26). In this study, nuclear overexpression of mdm2 products was observed in 59.2% of cases. We found that 17 tumors coexpressed both mdm2 and p53 products. These cases had an aggressive clinical course, as revealed by their association with the development of lymph node metastases. This phenomenon has been previously described for soft-tissue sarcomas (27) and head and neck carcinomas (28). In these studies, coexpression of mdm2 and p53 was associated with decreased survival (27) and advanced disease stage (28). The high incidence of mdm2 overexpression observed in this study might be explained by elevated levels of wild-type p53 in response to prolonged bilharzial infestation and potential development of DNA damage. In this context, mdm2 overexpression should be regarded as an oncogenic event. Due to DNA degradation we were unable to substantiate potential mdm2 amplifications that may occur in BBC.

Another downstream effector protein of p53 is p21/WAF1 (29). The p21/WAF1 gene is a member of the cyclin-dependent kinase inhibitors (29, 30). The rate of p21/WAF1 mutations seems to be very low (31, 32), and it has been postulated that its regulatory role rests at the expression level (31). In the present study, 87 of 121 (72%) evaluable cases showed p21 overexpression. The fact that 15 of 25 p53-positive cases had a p21-positive phenotype suggests that either altered p53 expression did not affect p53 function or that the induction of p21 was produced by a p53-independent pathway. In view of the number of cases displaying a mdm2-positive phenotype, the second hypothesis is favored. Furthermore, it has been shown that serum or individual growth factors, such as platelet-derived
growth factor, fibroblast growth factor, and epidermal growth factor can induce p21 in p53-deficient cells (33).

The high proliferative index and the infrequent apoptosis observed in these neoplasms are in accordance with their aggressive clinical behavior. The statistically significant association of high Ki67 proliferative index with clinicopathological parameters of poor outcome, such as deep muscle invasion and development of metastatic disease, support this concept. Moreover, the significant correlation between high Ki67 index and mdm2 overexpression further sustains its potential oncogenic role in this setting. In view of these results and of the enhanced proliferative activity in BBC, it is our postulate that the induction of p21/WAF1 may be the result of the presence of overexpressed cellular mitogens such as basic fibroblast growth factor and epidermal growth factor. In this regard, high levels of several growth factors have been reported to occur in bladder cancer (34).

In summary, data from the present study reveals a lower frequency of p53 nuclear overexpression in BBC when compared to that reported for cTCC. Nevertheless, tumors with p53 alterations have a greater propensity to progress. The prominent number of cases displaying an mdm2-positive phenotype suggests that this may be an early incident in BBC and should be regarded as a potential oncogenic phenomenon. This is supported by the significant correlation between high Ki67 proliferative index and mdm2 overexpression. The association of an aggressive clinical course with the coexpression of both p53 and mdm2 products might be viewed as a cooperative effect that develops in tumor progression. Finally, the biological implications of the disbalance observed between the rate of proliferation and apoptosis in these neoplasms may underline their aggressive clinical course, including lack of response to distinct treatment strategies. In addition to p53, pRB exerts a crucial role in the control of the cell cycle, mainly at the G1 check point. Deregulation of both p53 and pRB has been reported to engender tumor cells with selective growth advantage and reduced response to programmed cell death (35). We are currently in the process of investigating the potential implications of alterations affecting the pRB control pathway in the context of the etiology and progression of BBC.

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