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ARTICLE

Human Papilloma Virus and p53 Expression in Bladder Cancer in Egypt: Relationship to Schistosomiasis and Clinicopathologic Factors

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The aim of the current study was to compare the role of p53 and human papillomavirus (HPV) in schistosomiasis-related and schistosomiasis-unrelated carcinoma of the urinary bladder. To achieve this aim, we investigated 114 bladder carcinomas for p53 oncoprotein expression by immunohistochemistry and for human papillomavirus by in situ hybridization technique. The results revealed that 64 tumors (56.1%) were schistosomiasis-associated. Sixty seven (58.8%) were transitional cell carcinomas and 32 (28%) were squamous cell carcinomas. The remaining 15 tumors (13.2%) included adenocarcinomas and sarcomatoid carcinomas. In both schistosomiasis-associated and non-associated carcinomas, p53 oncoprotein expression was significantly higher in poorly differentiated tumors. However, it was significantly higher in locally more invasive tumors in the schistosomal carcinomas only. HPV types 16/18 could be detected in 1 of the 114 bladder carcinomas (0.95%), which was schistosomiasis-related squamous cell carcinoma in situ. These results suggest that p53 immunohistochemistry can be a prognostic factor in both schistosomal and nonschistosomal bladder cancer. More importantly, HPV does not seem to play a role in the pathogenesis of either type of bladder cancer in our country. (Pathology Oncology Research Vol 12, No 3, 173–178)

Key words: Egyptian patients, bladder carcinoma, schistosomiasis, p53, HPV

Introduction

In Egypt, carcinoma of the urinary bladder is the most common cancer in men, accounting for about half of all male malignancies. This type of cancer is of great clinical significance because its etiology, pathology and biological behavior differ considerably from that seen in Europe and the United States. The disease affects the Egyptian patients at a relatively younger age, is usually associated with schistosomal infection and shows a higher mortality rate.[1,2] From the pathologic point of view, bladder cancer in Egyptian patients is characterized by high frequency of squamous cell carcinoma due to schistosomiasis which induces squamous metaplasia of the urothelium.[3-5] Recently, however, a relative increase in the frequency of transitional cell type in schistosomiasis-associated bladder cancer has been noted.[6] This may indicate the implication of other etiologic factors in Egyptian bladder cancer patients.

Numerous studies have claimed that inactivation of the tumor suppressor gene p53 plays a role in the pathogenesis of bladder cancer.[7-13] It is worth mentioning that all of these studies were limited to transitional cell carcinoma which was not schistosomiasis-associated. Analysis of p53 in schistosomal and/or squamous cell carcinoma of the urinary bladder is confined to few studies, all of which from non-Egyptian authors.[14-18]

On the other hand, the role of human papillomavirus (HPV) in the pathogenesis of carcinoma of the urinary bladder has been extensively investigated with controversial results.[19-30] Only one of these studies included schistosomal cases from South Africa.[26] Another study was published during our work reporting a high incidence of HPV in bladder cancer in Egypt.[31] This contradicts with our results which will be discussed.

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In the present study we conducted a combined analysis of p53 protein and HPV in carcinoma of the urinary bladder in a cohort of Egyptian patients. Furthermore, p53 immunohistochemistry was correlated to the schistosomal status as well as other available prognostic factors.

Materials and Methods

Specimen collection

One hundred and fourteen specimens of bladder carcinoma were obtained from patients admitted at the university hospital. They were 102 males and 12 females. The patients' age varied from 38 to 69 years with a mean age of 50.8±8.32 years. Tumor specimens were taken by cystectomy in 103 cases and cystoscopy in 11 cases. Only cystoscopic biopsies containing muscle tissue were included, so that muscle invasion by the tumor could be assessed.

Histopathology

All 114 tumor specimens were fixed in 10% buffered formalin and embedded in paraffin. Hematoxylin and eosin (H&E) stained sections were prepared and examined to assess (1) the presence of schistosoma ova within the tumor tissue or adjacent non-neoplastic bladder tissue; (2) tumor type according to the World Health Organization;[32] (3) tumor grade according to the International Society of Urological Pathology;[33] and (4) tumor staging according to the degree of infiltration of the bladder wall, perivesical fat and adjacent structures.[34]

p53 immunohistochemistry (IHC)

Paraffin-embedded tumor tissue sections were cut on poly-L-lysine-coated slides. Tissue sections were deparaffinized with xylene and rehydrated by decreasing concentrations of ethanol. After blocking the endogenous peroxidase activity, the slides were put in citrate solution and treated in a microwave for 10-15 minutes. After application of protein blocking, the tissue sections were incubated for one hour with a mouse monoclonal antibody to p53 (1801, BioGenex, San Ramon, CA, USA) diluted 1:100. The procedure of IHC was completed using the avidin-biotin peroxidase technique[35] and the supersensitive detection kit (BioGenex); biotinylated anti-immunoglobulin was applied for 20 min, then peroxidase-labeled streptavidin was added for 20 min. The reaction product was visualized by adding the chromogen DAB (diaminobenzidine). All incubations were done at room temperature in a humidity chamber. A tissue section from a case of breast carcinoma known to be p53-positive was used as a positive control. For each case, a negative control was applied by following all steps of IHC except for replacement of the primary antibody by mouse IgG.

Positive immunoreaction for p53 oncoprotein was indicated by the presence of brownish nuclear staining of the malignant cells. This positivity was graded semiquantitatively, according to the percentage of positive cells on 0-4+ score.[36] Moreover, it was automatically quantitated by CAS 200 image analyzer (Becton Dickinson, San Jose, CA, USA), and the software program supplied by the company. This was measured as the percentage of positively stained nuclear area relative to the total nuclear area.

HPV in situ hybridization (ISH)

Four-µm-thick sections were cut from the paraffin blocks on poly-L-lysine-coated slides. Tissue sections were kept overnight at 60°C and then deparaffinized and rehydrated as in case of p53 IHC. Nucleic acids were unmasked by incubation with proteinase K at 37°C for 15 min. ISH technique was performed according to Krager[37] by using biotinylated HPV probes (types 6/11, 16/18 and 31/33) and supersensitive ISH detection kit (BioGenex). The tissue sections were incubated with the biotinylated probe solution at 95°C for 15 min to allow denaturation of the target DNA and probe, and then allowed to hybridize at 37°C for 2 hours. Visualization of the hybridized probe was achieved by incubating the sections with anti-biotin, followed by biotin-conjugated anti-immunoglobulin, each for 20 min at 37°C. Finally, peroxidase-labeled streptavidin was added for 20 min at 37°C. The sections were incubated with the chromogen substrate (DAB) and counterstained with hematoxylin. The positive control consisted of cervical tissue known to contain HPV by polymerase chain reaction. Negative controls were prepared by following all steps of ISH, except for replacement of the HPV probe by a negative DNA control probe (unrelated DNA sequence). Any case with definite nuclear brownish staining of the epithelial cells was considered positive, irrespective of the number of positive cells.

Statistical analysis

The relationship between p53 protein immunostaining results and other available clinicopathologic parameters was assessed by univariate analysis, using chi-square test. Multivariate analysis with backward variable selection was performed using logistic regression method in order to determine whether any of the factors tested could be independently associated with p53 expression. In addition, the semiquantitative grading of p53 immunostaining was correlated to its computerized quantitative measurement by applying Spearman's rank correlation coefficient.

Results

Histopathologic data

Schistosoma ova were detected in 64 of the 114 bladder carcinomas (56.1%). Sixty-seven were transitional cell carcinomas (58.8%), 32 squamous cell carcinomas (28.0%), one of which was in situ, 9 adenocarcinomas (7.9%), and 6 sarcomatoid carcinomas (5.3%). Muscle invasion (T2 + T3 + T4) was observed in 94 tumors (82.5%).

p53 immunohistochemistry (IHC)

Sixty-two of the 114 bladder carcinomas (54.4%) revealed positive nuclear staining for p53 protein (*Figures 1-3*). Correlation between visual semiquantitative and computerized quantitative analysis of p53 results showed a significant linear relationship (r=0.81, P=0.01) (*Figure 4*).

Thirty-six of the 64 schistosomiasis-related bladder carcinomas (56.3%) and 26 of the 50 non-schistosomal carcinomas (52.0%) revealed nuclear p53 protein expression. This difference was not statistically significant. *Tables 1* and *2* show the relationship between p53 protein expression and other investigated factors in schistosomal and non-schistosomal tumors, respectively. In both groups of bladder carcinomas, p53 was more significantly expressed in high-grade (grade 3) than in low-grade (grades 1 and 2) tumors as shown by univariate and multivariate analyses. No association was found with other variables in the schistosomal group.

In the non-schistosomal bladder carcinomas, p53 was more significantly expressed in tumors with vs. those without muscle invasion. No relationship could be obtained between p53 positivity and the age or sex of the patients, or the tumor type.

HPV in situ hybridization (ISH)

One of the 114 bladder carcinomas showed positive nuclear staining for HPV. It was schistosomiasis-associated squamous cell carcinoma in situ (*Figure 5*).

Discussion

In Egypt and other parts of Africa, bladder cancer is the first or second among all types of cancer in males, with a peak age of 50±5 years. It is characterized by high frequency of SCC.[2,5] This contrasts with the pattern reported in the Western literature where it is the fifth to seventh most common cancer in males with a peak age in the sixth or seventh decade.[38] Pathologically, SCC is much less frequent in Western than in African countries.[1] These differences in the incidence and pathology of bladder cancer between first and third world are mainly attributed to schistosomiasis which is the most frequent endemic parasitic disease in Egypt and many other African areas.[6,39]

Although p53 expression in bladder cancer has been extensively studied by Western authors,[7-18] no international reports from Egypt have been available until starting

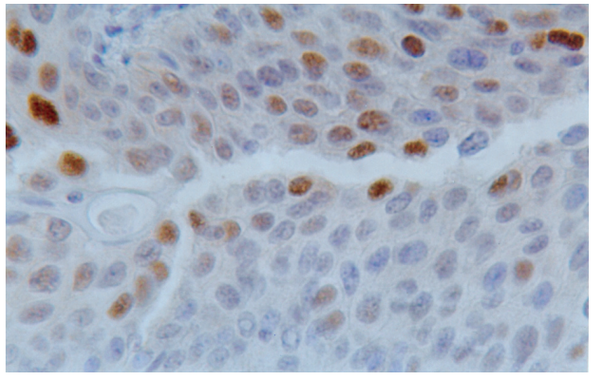

Figure 1. *A case of transitional cell carcinoma of the urinary bladder, showing positive nuclear immunostaining for p53 oncoprotein (×250)*

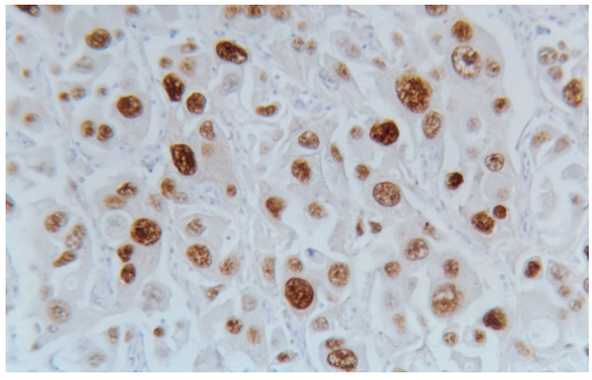

Figure 2*. A case of squamous cell carcinoma of the urinary bladder, showing positive nuclear immunostaining for p53 oncoprotein (×250)*

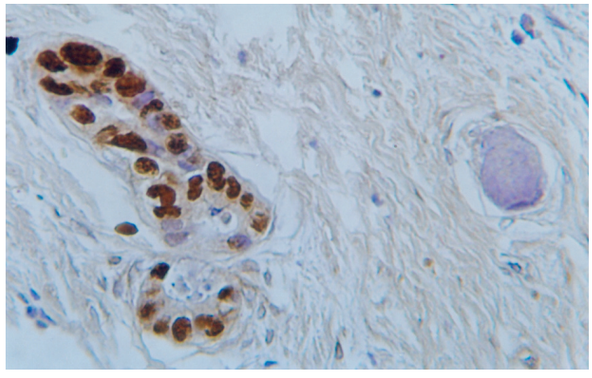

Figure 3. *A case of adenocarcinoma of the urinary bladder with shistosoma ovum (on the left side), showing positive nuclear immunostaining for p53 oncoprotein (×250)*

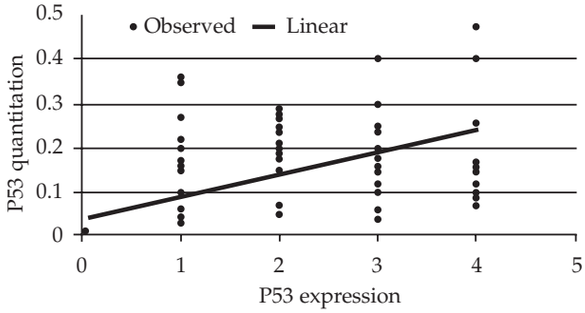

Figure 4. *Linear relationship between semiquantitative and computerized assessment of p53 expression in urinary bladder carcinoma (r=0.81, P=0.01)*

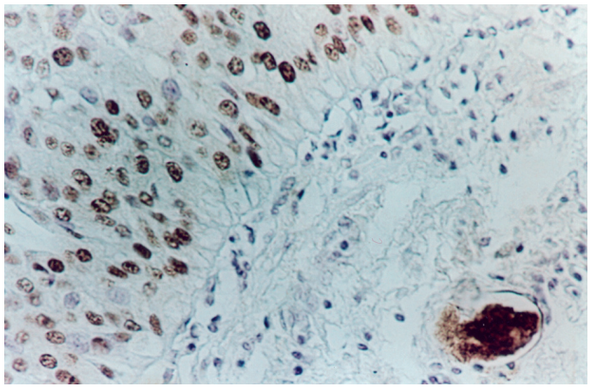

Figure 5. *A case of squamous cell carcinoma in situ showing positive nuclear immunostaining for HPV (ovum is present in the subepithelium) (ISH, ×250)*

our work. Similarly, the association of human papilloma virus (HPV) and non-schistosomal bladder cancer has been investigated in numerous studies,[19-30] however, the role of HPV in the pathogenesis of schistosomiasis-associated bladder carcinoma has not yet been reported except for the study of Cooper et al, which investigated South African cases.[26]

The present study compared p53 and HPV expression in schistosomal and non-schistosomal bladder cancer in Egyptian patients. Furthermore, the relationship between these factors and other pathologic parameters were assessed. The results revealed that p53 protein immunostaining was demonstrated in 56% of bladder carcinomas associated with schistosomiasis and 52% of those without schistosomiasis. These figures are comparable to those obtained by Kamel et al (55% and 50%, respectively).[15] However, Chaudhary et al showed that 76% of the schistosomiasis-associated bladder carcinomas expressed p53 immunoreactivity.[18] This higher frequency of p53 positivity might be due to inclusion of larger number of poorly differentiated tumors which are known to show more p53 protein expression.[40-42]

The absence of a significant difference between schistosomal and non-schistosomal bladder cancer regarding the frequency of p53 immunopositivity is in agreement with the study of Kamel et al which was the only report investigating such relationship.[15] This result suggests that p53 mutational events underlying bladder carcinogenesis may not be modulated by schistosomiasis.

Poorly differentiated bladder carcinomas revealed a significantly higher frequency of p53 positivity in tumors both with and without schistosomiasis. This finding agrees with previous reports.[40-42] It suggests that schistosomiasis does not affect the role of p53 in favoring the evolution of high-grade bladder cancer.

p53 expression was significantly more frequent in deeply invasive non-schistosomal bladder cancer, which agrees with other authors who proposed that p53 protein accumulation is associated with progression of bladder cancer.[15,16,18] We and Kamel et al[15] found no association between p53 and depth of tumor invasion in the schistosomiasis-associated carcinomas. This observation suggests that schistosomiasis may modulate the effect of p53 on the process of disease progression.

The role of HPV in bladder carcinoma is a subject of controversy. Numerous studies have analyzed the presence of HPV in non-schistosomal bladder cancer.[19-25,27-30] By using in situ hybridization technique, we could detect HPV types 16 and 18 in only one of the 114 bladder carcinomas. In fact, this low frequency of HPV positivity in our cases

Table 1. **Relationship between p53 protein expression and other factors in schistosomal bladder carcinoma**

Factor	*p53-positive*	*Univariate P*	*Multivariate*		
			RR	*95% CI*	*P*
Sex					
Male	32/56	NS	1.6	0.2-13.7	NS
Female	4/8				
Age					
≤ 50	9/13	NS	0.3	0.1-1.4	NS
> 50	27/51				
Tumor type					
TCC	16/32	NS	0.5	0.3-1.1	NS
SCC	10/19				
Tumor grade (TCC & SCC)					
Low (1&2)	12/31	0.04	2.2	1.1-4.4	0.03
High (3)	14/20				
Muscle invasion					
Negative	3/6	NS	0.9	0.3-2.8	NS
Positive	33/58				

TCC: transitional cell carcinoma, SCC: squamous cell carcinoma, RR: relative risk, CI: confidence interval

Table 2. **Relationship between p53 protein expression and other factors in schistosomal bladder carcinoma**

Factor	*p53-positive*	*Univariate P*	*Multivariate*		
			RR	*95% CI*	*P*
Sex					
Male	23/46	NS	3.9	0.3-55.9	NS
Female	3/4				
Age					
≤ 50	15/27	NS	0.5	0.1-2.1	NS
> 50	11/23				
Tumor type					
TCC	15/35	NS	0.8	0.3-0.9	NS
SCC	9/13				
Tumor grade (TCC & SCC)					
Low (1&2)	13/30	0.007	2.7	1.2-5.8	0.02
High (3)	11/18				
Muscle invasion					
Negative	2/14	0.0005	4.7	1.8-12.4	0.002
Positive	24/36				

TCC: transitional cell carcinoma, SCC: squamous cell carcinoma, RR: relative risk, CI: confidence interval

is expected since the Egyptian traditions limit sexual relationship with multiple partners which is the most common cause of HPV infection. The low frequency of HPV positivity in schistosomiasis-associated bladder carcinomas agrees with Cooper et al who failed to demonstrate HPV in 25 schistosomal squamous cell carcinomas by using in situ hybridization technique.[26] Our results and those of Cooper et al suggest that an alternative pathway, other than HPV, can be involved in the pathogenesis of schistosomal bladder cancer. An interesting recent study revealed that multiple factors could play a role in schistosomiasis-associated bladder carcinogenesis.[6] Examples of these factors are nitroso compounds, increased activity of carcinogen metabolizing enzymes and increased DNA damage. Unexpectedly, Khaled et al reported a high frequency of HPV in Egyptian bladder cancer patients (53% in schistosomal cases) by in situ hybridization.[31] These discrepancies between our study and Cooper et al[26] on one hand and that of Khaled et al[31] on the other hand may be explained by the variability in the sensitivity of HPV detection depending on sample fixation, DNA preparation conditions and patient population.[43]

In conclusion, the current study showed that both in schistosomiasis-associated and non-associated bladder carcinomas, p53 immunopositivity was significantly higher in poorly differentiated tumors. On the other hand, it was more frequently expressed in deeply invasive than in non-invasive cases only in non-schistosomal carcinomas. More importantly, HPV seems to play no role in the pathogenesis of schistosomal or non-schistosomal bladder cancer in our country. However, further studies are still recommended to confirm this concept.

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