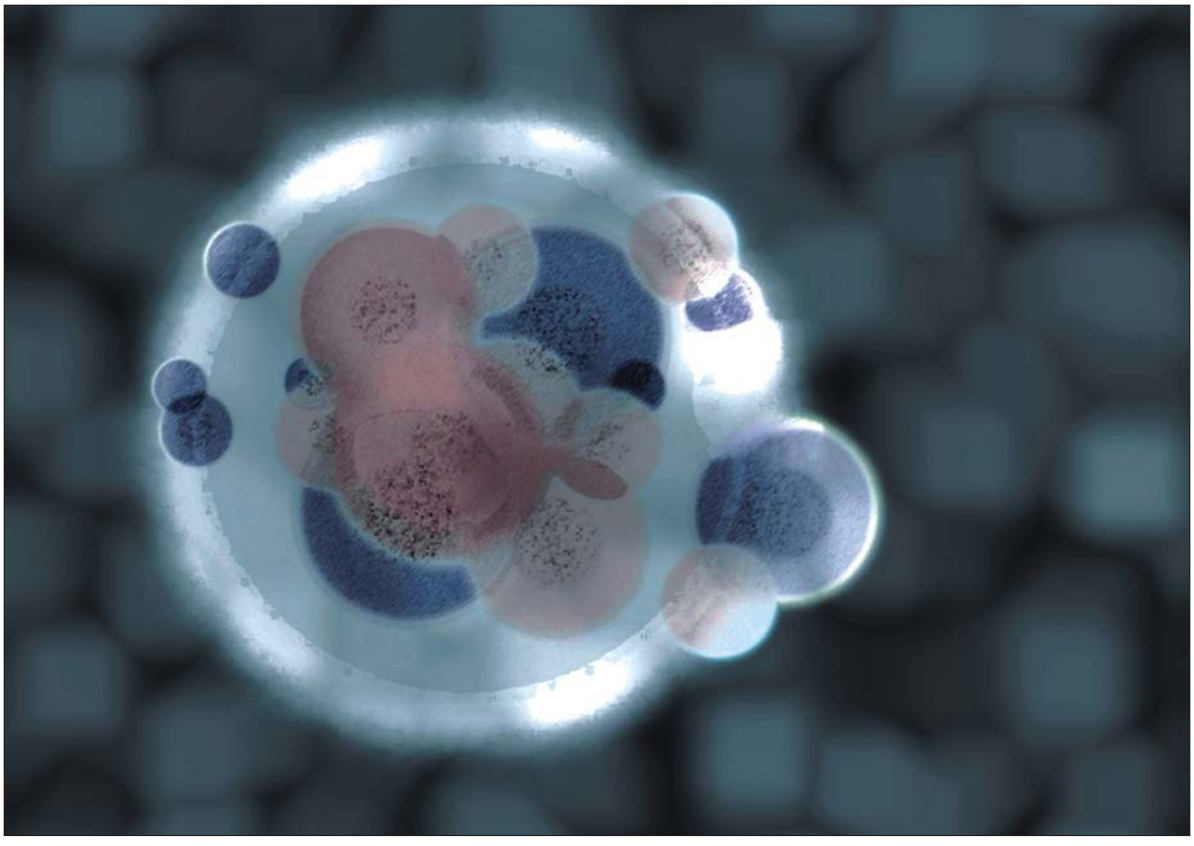

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Immunohistochemical Study of Inducible Nitric Oxide and its Prognosis in Schistosomasis Associated Urinary Bladder Carcinoma

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Abstract: The aim of this study was to examine iNOS expression in schistosomasis associated bladder carcinoma. iNOS expression was determined by immunohistochemisty. iNOS expression was found in almost 57.8% of bladder cancer patients. The significant correlation between the expression of iNOS and tumor parameters was present ($p = 0.00948$, 0.02702, 0.00013, 0.00006 and 10^{-6} for sex, tumor grades, tumor stage, DNA ploidy, lymph node status and tumor recurrence respectively. The overall survival for patient was 62.22% where the 5 year disease-specific survival was significantly with iNOS, tumor grade, tumor stage, DNA ploidy and lymph node status ($p = 10^{-6}$, 0.0026, 0.0072, 0.0119 and 10^{-6}, respectively). In multivariate analysis, the three markers, iNOS, tumor grade and lymph node status remained the prognostic in bladder cancer and the more significant was iNOS. Present results suggest that iNOS expression in schistosomasis associated urinary bladder carcinoma may predispose to cancer recurrences and confirm that iNOS could be considered a good prognostic factor.

Key words: Bladder cancer, prognosis, iNOS, schistosomasis

INTRODUCTION

Nitric oxide (NO) is generated by a family of nitric oxide synthase enzymes (NOS). The endothelial and neuronal NOS isoforms are expressed constitutively and require the presence of intracellular Ca^{2+} and calmoduline, generating nanomolar levels of NO. Inflammatory cytokines and/or bacterial products usually activate the inducible NOS (iNOS), generating large amounts of NO (Knowles and Moncada, 1994). iNOS is also expressed in a variety of tumor cells and it has been suggested to play a controversial role in tumor biology, either promoting or inhibiting tumor growth, depending on the tumor model levels of NO delivered, genetic background and cell type all of which can determine NO sensitivity (Eijan *et al*., 1998; Wang *et al*., 2001a, b). For example, it has been reported that continuous NO production may be involved in the inflammatory process associated with the appearance of many human malignancies, including bladder cancer (Ohshima and Bartsch, 1994; Balkwill and Mantovani, 2001; O'Byrne and Dalgleish, 2001). Besides, it is known that N-nitroso compounds, of which NO is a precursor, induce a significant number of cancers (Mirvish, 1995). On the other hand, mechanisms involved in bacterial or tumor cell death are mediated by cells of the immune system such as macrophages, which can produce high levels of NO (Wheeler *et al*., 1997; Zhuang *et al*., 2000). Bladder carcinoma is the fourth most prevalent type of cancer in men in Wetstern society. The main aetiological factors are exposure to industrial carcinogens and cigarette smoking. Another major aetiological factor is infestation by the parasite *Schistosoma haematobium*. This parasite is endemic in Africa and the Middle East. The adult worms reside in the venules of the urinary bladder where they lay the eggs. These elicit a pronounced inflammatory reaction and fibrosis in the bladder wall and initiate hyperplasia of the bladder mucosa, followed by neoplastic transformation (Shochina *et al*., 2001).

It has been postulated that NO produced by iNOS in the inflammatory process in schistosomal infection causes damage to genomic DNA and may be the mechanism of induction of carcinogenesis in bladder carcinoma in infected individuals. Recently, iNOS expression has been documented in the inflammatory cells and in isolated tumour cells in bladder carcinoma, but the expression of NOS in schistosomal bladder cancer has not been studied. The inducible isoform of NOS was identified in the human urine pellet and NOS activity was increased in urinary tract infections and decreased in interstitial cystitis (Shochina *et al*., 2001).

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In our knowledge, there are many reports showing expression of NOS in solid tumors, but only few show iNOS expression in human bladder carcinoma. In the present study, we examined iNO expression and activity in schistomasis associated human urinary bladder cancer and correlated this iNO expression with tumor parameters of this cancer.

MATERIALS AND METHODS

Tumor samples were obtained when total cystectomy of urinary bladder were performed at the Department of Urology of the Urology and Nephrology Center, Mansoura University, Egypt. Formalin-fixed, paraffin-embedded sections from ninety bladder tissues were used to analyze iNOS distribution. Immunohistochemical staining was performed using an avidin-biotin peroxidase complex. Briefly, samples were treated with 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Staining of formalin-fixed tissues requires boiling tissue sections in 10 mM citrate buffer, pH 6.0, (NEOMARKERS' Cat. #AP-9003), for 10-20 min followed by cooling at RT for 20 min. The slides were preincubated with normal goat serum (1:10) (Vector Laboratories, Burlingame, CA,) for 10 min and then with human specific polyclonal rabbit anti-iNOS antibody (Ab-1, Lab. Vision, NeoMarkers, USA), Using antibody dilution at 10-20 μg mL^{-1} for 60 min at room temperture. The sections were further incubated with biotinylated goat anti-rabbit IgG diluted 1:500 (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min, followed by incubation with peroxidase-conjugated streptavidin diluted 1:3000 in phosphate- buffered saline for 15 min. The peroxidase reaction was performed using 0.02% 3, 3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide and counterstaining was performed with hematoxylin for 1 min. As negative control, the primary antibody was omitted. The histopathological and DNA ploidy data were available such as tumor stages, tumor grades, lymph nodes status, tumor recurrence, follow up and the tumor ploidy.

Statistical analysis: Pearson's χ^2 test was used to evaluate the interrelations between iNOS positivity overexpression, histological grade, stage and lymph node involvement; the disease free survival curves of the TCC patients were estimated using the Kaplan-Meier method. Statistical analyses of the differences between curves were performed using the log-rank test. Variables that significantly influenced survival (p = 0.05) in univariate analysis were entered into a multivariate Cox regression model. The significance level for all analyses was set at 0.05. The statistical analysis was performed using the statistical package for social sciences software package, version 6.1 (SPSS Inc.139 Chicago, III).

RESULTS

Sixty-five males and twenty-five females were identified ranging in age from 28 to 70 years (median 49 years). Inducible Nitric Oxide Synthase (iNOS) expression immunoreactive cells was in 52/90 cases (57.8%). The correlation iNOS expressions and tumor parameters was shown in Table 1 and Fig. 1 and 2, where the iNOS expression was significant correlated with sex, tumor grade, tumor stage, DNA ploidy, nodal status and incidence of local recurrence (p-value = 0.00948, 0.02702, 0.00013, 0.00006 and 10^{-6}, respectively). The highly expression of iNOS was present in males (43 cases), GII (37 cases), invasive stage (41 cases), DNA aneuploidy (43 cases), positive lymph node (41 cases) and in positive recurrence (32 cases) from all iNOS positive cases (52 cases).

Prognosis in patients with univariate analysis: Kaplan-Meier estimate of the overall disease-free survival at 5 years was 62.22% of all cases (90). Treatment was high during the first 3 years and then declined thereafter, where the follow up period ranged from 1 month to 165 moths

Table 1: Correlation between the expression of iNOS with tumor parameters

	iNOS expression		
Parameters	Negative		Positive
Sex			
Male	22		43
Female	16		9
p-value		0.00948	
Grade			
GI	20		13
GII	17		37
GIII	1		2
p-value		0.02702	
Stage			
pT1	6		1
pT2	22		15
pT3	10		36
p-value		0.00013	
DNA ploidy			
Diploid	22		9
Aneuploid	16		43
p-value		0.00006	
Lymph node status			
Negative	31		11
Positive	7		41
p-value		10^{-6}	
Recurrence			
Negative	36		20
Positive	2		32
p-value		10^{-6}	

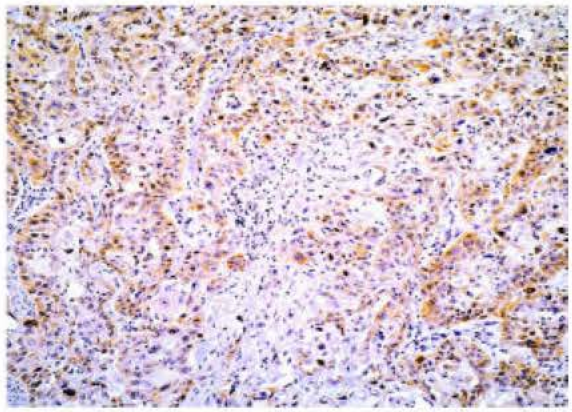

Fig. 1: Transitional cell carcinoma of the urinary bladder showing cytoplasmic stained with anti-EGFr antibody (avidin-biotin immunoperoxidase X 100)

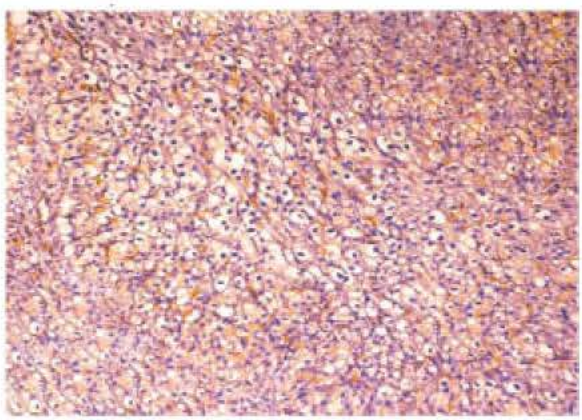

Fig. 2: Transitional cell carcinoma of the urinary bladder showing cytoplasmic stained with anti-MMP-2 antibody (avidin-biotin immunoperoxidase X 250)

(mean 65.7 months). The following variables: iNOS, tumor graded, P-stage, DNA ploidy, lymph node status were significant correlated to survival data (p value = 10^{-6}, 0.026, 0.0072, 0.0119, 10^{-6}, respectively); as shown in Table 2 and Fig. (3-5). The survival rates in negative cases of iNOS was better (97.74%) than in positive cases (33.33%). Also, the survival rates in low grade (GI) cases (81.21%) and superficial tumor stage P1 (100%), diploid DNA cases (77.16) and negative lymph node cases (87.90%) were better than in high grades (GII and GIII) cases and invasive tumor stage (P2 and P3), aneuploidy DNA and positive lymph nodes cases.

Prognosis in patients with univariate analysis: The five factors that had a significant impact on survival with the univariate analysis were further evaluated by the Cox's regression model. With such analysis, iNOS, tumor grade and lymph node status, remained the only factors that had a most statistically significant impact on survival as shown in Table 3.

Table 2: Survival relation to iNOS expression, Grades, Tumor stages, DNA ploidy and lymph node status

Parameters	No. of patients	5 year survival (%)	p-value
iNOS			
Negative	38	97.74	
Positive	52	33.46	$<10^{-6}$
Tumor grades			
I	33	81.21	
II	54	52.57	0.0026
III	3	33.33	
Tumor stage			
pT1	7	100.00	0.0072
pT2	37	74.00	
pT3	46	47.23	
DNA			
Diploid	31	77.16	0.0119
Aneuploid	59	52.58	
Lymph node status			
Negative	42	87.90	$<10^{-6}$
Positive	48	37.47	

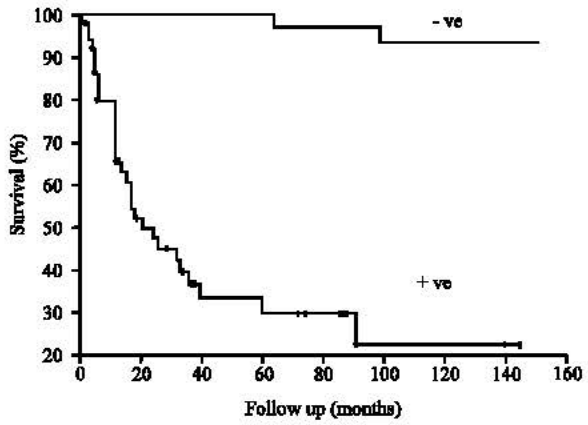

Fig. 3: Survival outcome related to iNOS

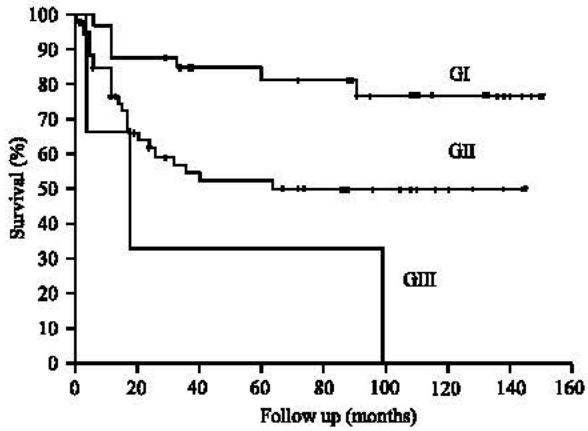

Fig. 4: Survival outcome related to tumor grades

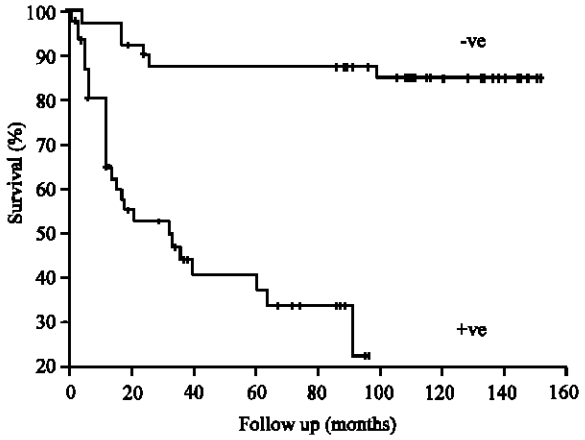

Fig. 5: Survival outcome relation to lymph node status

Table 3: Results of proportional hazard analysis (Cox regression) of disease-free survival

Parameter	Regression estimate	Standard error	p-value
iNOS			
Negative	0	-	
Positive	2.9550	0.8025	0.0003
Grade			
I	0	-	-
II	1.9986	0.7100	0.0049
III	1.1907	0.6251	0.0568
LN			
Negative	0	-	-
Positive	1.1947	0.5199	0.0216

DISCUSSION

The role of NO in tumor biology is still unclear. NO can have a dual role in tumor progression, since it can act as pro-as well as anti-tumor promoter, depending on its concentration, timing of secretion or cell type (Jenkins *et al*., 1995). The important bulk of information about NOS relevance in tumors has appeared in the last decade. In human gynecological cancers (ovarian and endometrial) and breast cancer, NOS activity has been reported to be directly associated with tumor grade (Thomsen *et al*., 1994 and 1995). Beside it was demonstrated that high iNOS expression is a bad prognosis marker in human colorectal carcinoma (Lagares-Garcia *et al*., 2001). Only few papers have reported the relevance of iNOS in bladder tumors showing no conclusive results (Swana *et al*., 1999; Shochina *et al*., 2001; Lin *et al*., 2003), but in our knowledge, no studies have reported the relevance of iNOS in schistosomasis associated bladder tumors. The abnormal expression of iNOS in the whole bladder, producing non-physiological concentrations of NO could generate a landscaping effect that helps in tumor development. As NO is the precursor of the known carcinogenic N-nitroso compounds (Mirvish, 1995; Kane *et al*., 1997), it is possible that supra-physiological levels of NO, generated in the bladder by enhanced activity of iNOS, may act as a carcinogenic stimuli. In addition, it is possible to hypothesize that the high output of NO may act as a growth promoter or growth survival factor for the remaining tumor cells after the treatment of bladder tumor patients (Wink *et al*., 1998). Here we show that, bladder cancer patients with positive iNOS tumors presented significantly earlier recurrences than those without iNOS expression.

In the present study, iNOS expression and activity were found in 57.8% but in Sandes *et al*. (2005) study, the expression was 50% in bladder cancer patients. By immunohistochemistry, heterogeneous iNOS staining was detected in tumor cells from superficial and invasive tumor. A follow up of patients after cystectomy ranged from 1 month to 165 months where it was shown tumor relapse in 35.6% but the tumor recurrence cases in positive expression of iNOS was 32/52 (61.5%). On the contrary, negative recurrences were observed in 36/38 (94.7%) of negative iNOS patients.

This study, the largest immunohistochemical study evaluating iNOS on bladder carcinoma published to date, demonstrated a statistically significant correlation between iNOS with sex, tumor grades, tumor stages DNA ploidy, lymph node status and tumor relapse. In previous studies, Llin *et al*. (2003) reported that a correlation between iNOS immunoreactivity and tumor grade in bladder carcinoma could not be verified. The inconsistent result may be attributed to the method of analysis or the sample size, also Wolf *et al*. (2000) were unable to show a clear correlation to tumor grade or stage in bladder carcinoma. Present results demonstrated iNOS expression was predictive factor of survival using the log-rank test ($p = 10^{-6}$), also these markers were independent predictive factors using multivariate analysis (Cox proportional hazard analysis). Present results also suggest that iNOS expression, tumor grade and lymph node status are a potentially useful tool for the selection of the postoperative observation strategy especially iNOS where it was the most significant parameter ($p = 10^{-6}$) and may be new valuable tumor markers for the screening, diagnosis and follow-up for patients with schistosomasis associated urinary bladder carcinoma. These results confirm that iNOS could be considered a good prognostic factor for schistosomasis associated urinary bladder cancer.

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