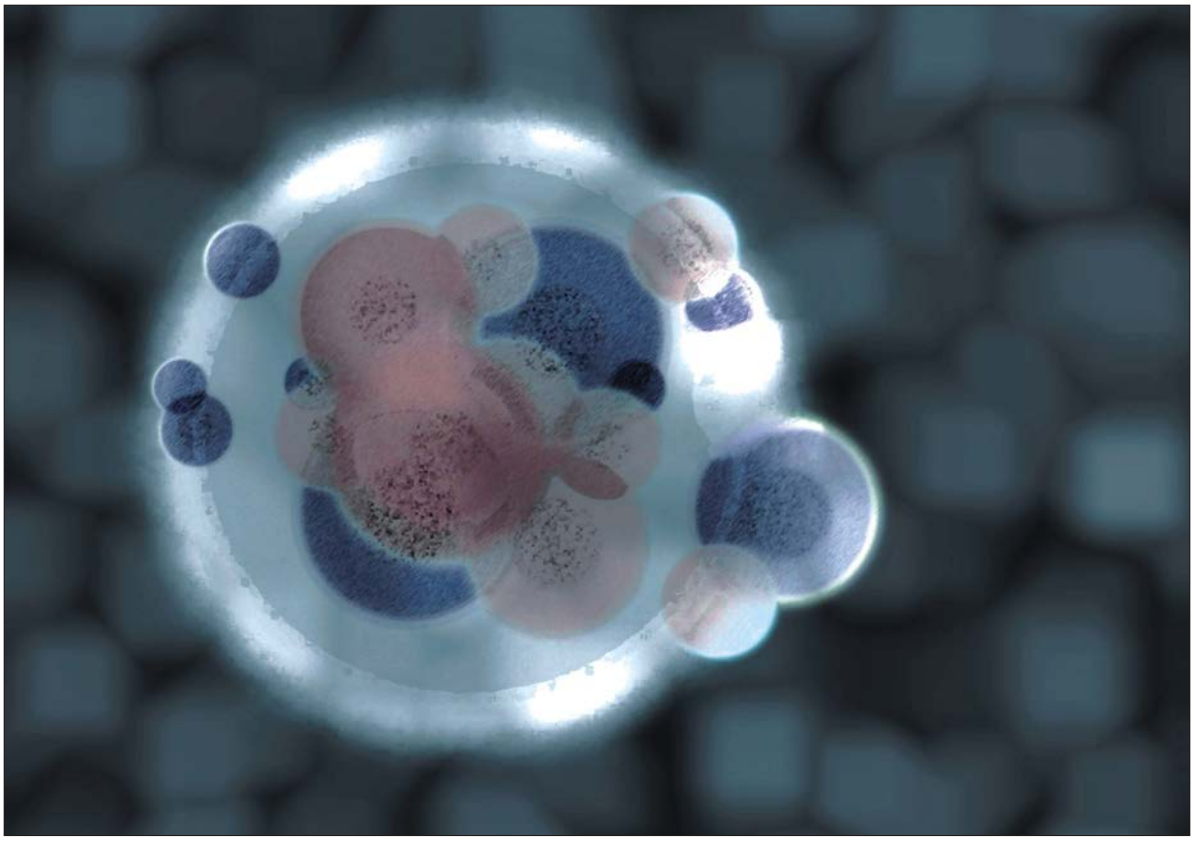

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Expressions of Epidermal Growth Factor Receptor, Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Bladder Carcinoma

[1]Ayman EL-Meghawry EL-Kenawy, [2]Attalla Farag El-kott,
[3]Mashael Mohammed Bin-Meferij and [4]Ezz Mohie El Gamal
[1]Institute of Genetic Engineering and Biotechnology, Menoufiya University, Egypt
[2]Department of Zoology, Faculty of Science, Fayoum University, Egypt
[3]Department of Zoology, Girls College of Education, Scientific Sections, Riyadh, K.S.A.
[4]Urology and Nephrology Center, Faculty of Medicine, Mansoura University, Egypt

Abstract: The aim of the present study was analyzed the expression of epidermal growth factor receptor (EGFR), matrix metalloproteinase-2 and 9 for evaluating their value as a prognostic markers in transitional bladder cancer specimens. The EGFr, MMP-2 and MMP-9 proteins were assayed semiquantitatively in 85 transitional bladder carcinomas of varying grade and stage. Specific monoclonal antibodies for EGFr, MMP-2 and MMP-9 were used to detect the antigens by immunohistochemical staining of paraffin-embedded tissue sections. Of the 85 specimens, 38 (44.7%) overexpressed EGFr, 38 (41.2%) MMP-2 and 38 (44.7%) MMP-9 proteins when three observers scored stained sections independently. The 5-year disease-specific survival was significantly with EGFr, MMP-2, MMP-9, ($p<10^{-6}$ for all three markers). In multivariate analysis, the three markers remained the prognositic in bladder cancer and the more significant was EGFr. The results of this study demonstrated that EGFr, MMP-2, MMP-9 proteins overexpression may be an independent prognostic biomarker for bladder cancer.

Key words: Bladder cancer, prognosis, EGFr, MMP-2, MMP-9

INTRODUCTION

Bladder cancer accounts for approximately 5% of all human cancers and represents 95% of all urothelial tumors (Izadifer *et al*., 1999). Transitional cell carcinoma (TCC) represents 90% of bladder cancer, while squamous cell carcinoma (SCC) accounts only 3-7% (Cotran *et al*., 1994). SCC of the bladder is uncommon in the developed countries; while in areas endemic for schistosomasis, this tumor is the commonest type of bladder malignancy (Tungekar and Linehan, 1998). The growth and development of cancer cells is thought to occur through multiple genetic events that cause fundamental changes in the pathways regulating cell differentiation, proliferation, survival and mobility.

Activation of the proto-oncogene encoding the epidermal growth factor receptor (EGFR), a growth factor receptor tyrosine kinase, may contribute to the transformation of cellular phenotypes and provide tumor cells with substantial growth and survival advantages. Over the last 20 years, elevated levels of the EGFR and its cognate ligands (which include EGF and transforming growth factor (TGF)-a) have been identified as a common component of numerous cancer types. In many cases aberrant EGFR activation, mediated primarily through changes in gene amplification and autocrine stimulation, appears to be an important factor in tumorigenesis, as well as an essential driving force for the aggressive growth behaviour of cancer cells (Nicholson *et al*., 2001). Overexpression of EGFR in bladder cancer has been widely reported and several studies have shown EGFR positivity to be associated with high tumor stage, tumor progression and poor clinical outcome. The mechanism by which EGFR expression is associated with poor prognosis is not entirely clear, although there is some evidence linking EGFR stimulated activation of activator protein-1 transcription factor with induction of matrix metalloproteinase activity (Colquhoun and Mellon, 2002).

Proteolytic degradation of extracellular matrix proteins by tumor cells is a critical step in the invasion of cancer. Matrix metalloproteinases (MMPs) play pivotal roles in the degradation of the extracellular matrix of tissues surrounding tumors. MMP-2 and MMP-9 have been detected in numerous malignancies and their elevated expression has correlated significantly with tumor invasion and poor prognosis in various carcinomas (Yasuyoshi *et al*., 2004). Also, several reports have indicated that MMP-2 and MMP-9 expression levels are

Corresponding Author: Dr. Ayman EL-Meghawry EL-Kenawy, Institute of Genetic Engineering and Biotechnology, Menoufiya University, Sadat City, P.O. Box 79, Egypt Fax: 020 48 2601266

associated with tumor stage and survival in patients with TCC (Nakanishi *et al*., 2000; Inoue *et al*., 2002). The rationale of the present study was to investigate the EGFr, MMP-2 and MMP-9 expressions in transitional urinary bladder cancer and correlate them with histopathological parameters.

MATERIALS AND METHODS

A total of 85 formalin-fixed, paraffin-embedded specimens of human bladder transitional cell carcinoma were used for this study in Genetic Engineering and Biotechnology Inst. Menoufiya University, Egypt. The tumors were representative of all grades (55 grade 1; 23 grade 2 and 7 grade 3) and of both superficial (1 pTa; 7 pT1) and muscle-invasive (77 Stage pT2 or greater) tumor stages. All experiments were performed after first obtaining consent from the local research and ethics committee according to the Medical Research Council regulations.

Immunohistochemistry: For immunohistochemistry, 4 μm paraffin sections were cut on slides coated with Histo-Grip (Zymed Laboratory, San Francisco, Calif, USA) and incubated overnight at 37°C. The slides were deparaffinized in a histological clearing agent (Histo-Clear, National Diagnostics, Atlanta, Ga) and hydrated. Then the slides were treated with 0.5% pepsin (2000 FIP-U/g, Merck, Darmstad, Germany) for 20 min at 37°C. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxidase in absolute methanol for 10 min and any nonspecific binding was blocked with 10% goat serum for 15 min. The avidin-biotin-immunoperoxidase technique was used as modified from the original description of Hsu *et al*. (1981). Monoclonal antibodies EGFR, MMP-2 and MMP-9 with dilution 1:50 for 30 min (Lab vision Warm Springs Blvd. Fremont CA 94539 USA) were used as the primary antibodies. Phosphate-buffered saline (0.01 M phosphate buffer, pH 7.5) with 1% bovine serum albumin was used as a buffer. The slides were incubated with primary antibodies for 30 min at room temperature in a humidified atmosphere. Afterward, immunohistochemical staining was continued using the Histostain-plus bulk kit (Zymed Laboratory, San Francisco, Calif, USA). As a secondary antibody, a biotinylated, human-absorbed, affinity-purified IgG was used. Peroxidase was introduced using streptavidin conjugate. The slides were washed thoroughly with phosphate buffered saline after each step in the procedure. The antibody reaction was visualized using a fresh substrate solution containing DAB substrate (Zymed Laboratory). The sections were counterstained with hematoxylin, dehydrated and mounted in Immu-Mount. For negative controls, the primary antibody was replaced with the mouse non-immuno IgG. Each set of staining also included a known positive control.

Evaluation of markers immunostaining scores: Three independent observers scored the immunostaining for EGFr, MMP-2 and MMP-9 in two repeatable experiments. The interobserver variability was low. The clinical data were not analyzed until the immunostaining scores were given. If 1% or more of the cells showed positive staining, the tumor was considered positive.

Statistical analysis: The overall and disease-free survival rates were determined by the Kaplan-Meier method (Kaplan and Meier, 1958) and the differences between the subgroups were analyzed using the log-rank test. p-values less than 0.05 were interpreted as statistically significant. Fisher's exact test for small samples was used to evaluate the association between markers expression and the tumor parameters.

RESULTS

Results of immunohistochemistry: The study included 85 cases of transitional cell urinary bladder cancer, 60 men and 25 women, with a median age of 45.98 years (range 30 to 64). At the postoperative pathologic examination, only 9 patients had lymph node metastasis. The mean follow-up period was 71.23 months (range 1 to 151). In Table 1, Positive immunoreactivity for EGFr, MMP-2 and MMP-9 was detected in 38 (44.7%), 35 (41.2%), 38 (44.7%) sections, respectively (Fig. 1-3). However, EGFr expression was significant correlation with DNA ploidy

Table 1: Clinicopathologic characteristics of tumor parameters and their relation to EGFr, MMP-2 and MMP-9

	EGFr		MMP-2		MMP-9	
	-ve	+ve	-ve	+ve	-ve	+ve
Grade						
I	35	20	38	17	38	17
II	10	13	8	15	7	16
III	2	5	4	3	2	5
p-value	0.09		0.02		0.003	
Stage						
Superficial	3	5	7	1	6	2
Invasive	44	33	43	34	41	36
p-value	0.23		0.3		0.1	
DNA						
Diploid	20	9	23	6	18	11
Aneuploid	27	29	27	29	29	27
p-value	0.005		0.0006		0.5	
Lymph node						
Negative	44	32	47	29	46	30
Positive	3	6	2	6	1	8
p-value	0.2		0.1		0.005	
Recurrence						
Negative	42	13	44	11	42	13
Positive	5	25	6	24	5	25
p-value	$<10^{-6}$		$<10^{-6}$		$<10^{-6}$	

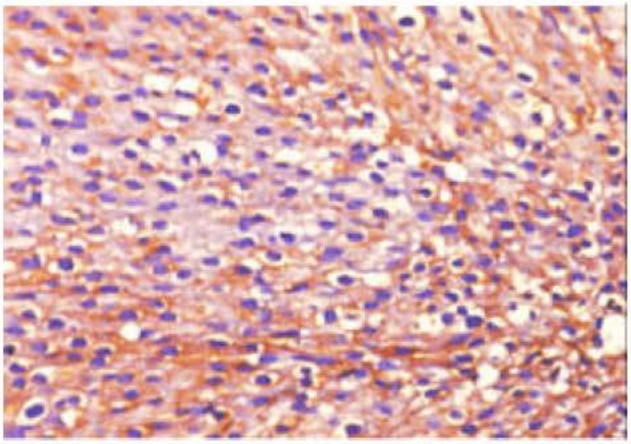

Fig. 1: Transitional cell carcinoma of the urinary bladder showing cytoplasmic staining with anti-EGFr antibody (avidin-biotin immunoperoxidase X 400)

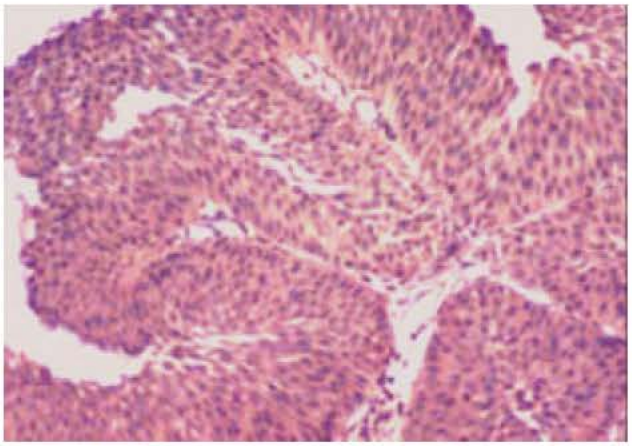

Fig. 2: Transitional cell carcinoma of the urinary bladder showing cytoplasmic staining with anti-MMP-2 antibody (avidin-biotin immunoperoxidase X 250)

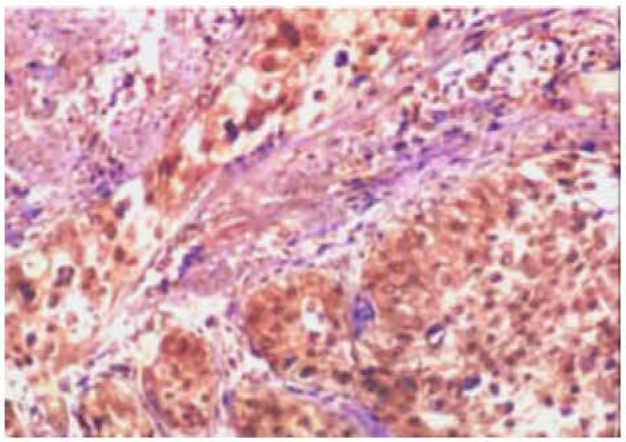

Fig. 3: Transitional cell carcinoma of the urinary bladder showing cytoplasmic staining with anti-MMP-9 antibody (avidin-biotin immunoperoxidase X 250)

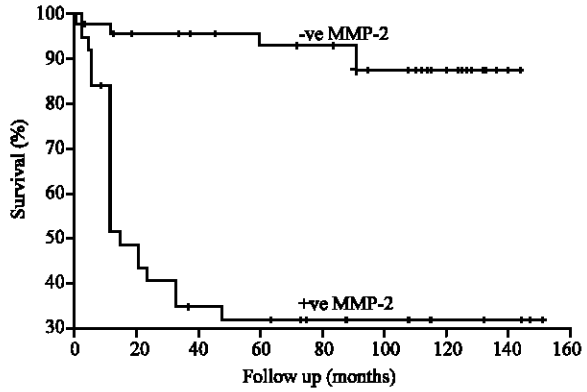

Fig. 4: 5-year survival of TCC patients in relation to tumor EGFr expression

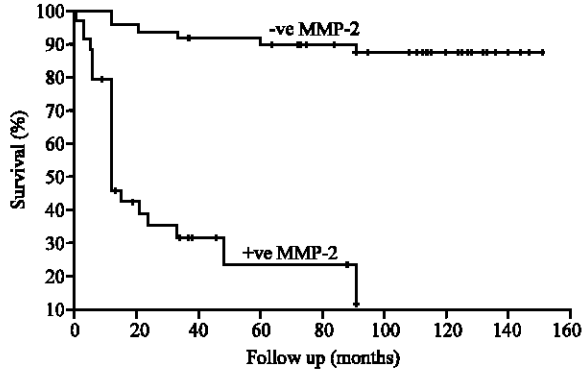

Fig. 5: 5-year survival of TCC patients in relation to tumor MMP-2 expression

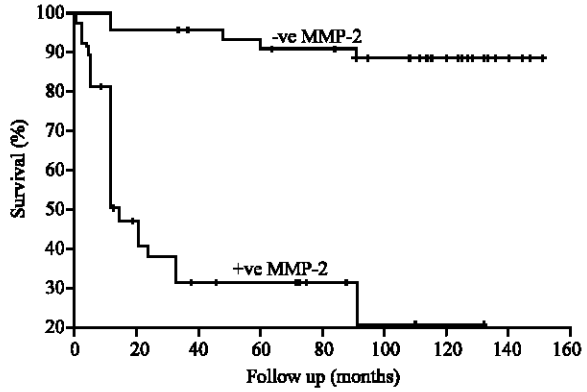

Fig. 6: 5-year survival of TCC patients in relation to tumor MMP-9 expression

and recurrence (p = 0.005, 10^{-6}, respectively), MMP-2 with grades, DNA ploidy and recurrence (p = 0.02, 0.0006, 10^{-6}, respectively) and in MMP-9 expression was significant correlated with tumor grades, lymph nodes involvement and recurrence (p = 0.003, 0.005, 10^{-6}, respectively).

Table 2: Results of proportional hazard analysis (Cox regression) of disease-free survival

	Number of patients	5-year survival (%)	p-value
Grade			
I	55	79.52	0.004
II	23	49.37	
III	7	28.57	
Stage			
Superficial	8	100	0.06
Invasive	77	62.34	
DNA			
Diploid	29	75.07	0.03
Aneupliod	56	44.16	
Lymph node			
Negative	76	72.63	0.0001
Positive	9	22.22	
EGFr			
Negative	47	93.18	$<10^{-6}$
Positive	38	35.31	
MMP-2			
Negative	50	89.96	$<10^{-6}$
Positive	35	24	
MMP-9			
Negative	47	93.41	$<10^{-6}$
Positive	38	31.65	

Prognosis in patients with transitional cell carcinoma (univariate analysis): Statistical analysis of outcome was performed with Kaplan-Meier survival curves. In univariate analysis, the 5 year overall survival rate was 64.71%. A statistically significant difference was found in the disease-specific survival of the patients with tumor grades, DNA ploidy, lymph nodes status and EGFr, MMP-2, MMP-9 positive or negative expressions for bladder carcinoma, p = 0.004, 0.03, 0.0001 and 10^{-6}, respectively, (Fig. 4-6). An unfavorable 5-year disease-specific survival rates were found in GIII (28.57%), aneuploid DNA (44.16%), positive lymph nodes for metastases (22.22%) and positive expressions of EGFr, MMP-2 and MMP-9 (35.31, 24 and 31.65%, respectively) as shown in Table 2.

Multivariate analysis: The six factors that had a significant impact on survival with the univariate analysis were further evaluated by the Cox's regression model. With such analysis, EGFr, MMP-2, MMP-9, remained the only factors that had a statistically significant impact on survival.

DISCUSSION

In this study, we examined the relationship between expression of EGFr and extracellular matrix degradative enzymes (MMP-2, MMP-9) and prognosis. the expression of three markers shows higher in positive recurrence than in negative cases.

EGFr, MMP-2 and MMp-9 essential role in tumor recurrence has already been clearly verified. Grade I, Superficial bladder carcinoma, DNA ploidy, negative lymph node, negative of EGFr, MMP-2 and MMP-9 have a good prognosis. The activation of the epidermal growth factor receptor (REGF) participates in oncogenesis by

inducing cell proliferation, cell mobility and angiogenesis and inhibiting apoptosis. This activation might be due to numerous abnormalities, including increased expression of its ligand. Although based on retrospective analyses with no standard technique of evaluation, the level of EGFr expression is a prognosis factor for several tumors (Penault-Llorca *et al*., 2003). Because of its role in oncogenesis and its prognostic value, EGFr might in the future become a therapeutic target.

This study, the largest immunohistochemical study evaluating MMP-2 and MMP-9 on bladder carcinoma published to date, demonstrated a statistically significant correlation between MMP-2 and MMP-9 with tumor grades but not with tumor stages. In previous studies, Grignon *et al.* (1996) reported that the biomarker MMP-2 did not correlate with bladder cancer grade or stage. The inconsistent result may be attributed to the method of analysis or the sample size. Kanayama *et al.* (1998) reported that reverse transcriptase-polymerase chain reaction analysis of the levels of MMP-2 correlated with cancer grade. Gerhards *et al.* (2001) also reported that MMP-2 and MMP-9 urinary excretion was associated with a high stage and grade of bladder carcinoma. Although MMP-2 and MMP-9 expression was a predictive factor of cause-specific survival using the log-rank test, these markers were not independent predictive factors using the Cox proportional hazard analysis (Yasuyoshi *et al*., 2004). Inoue *et al.* (2002) also reported that MMP-2 and MMP-9 were predictors of survival using univariate analysis but that neither were independent predictors in the multivariate analysis. Furthermore, synthetic MMP inhibitors had no therapeutic benefit in Phase III trials of several carcinomas. The expression of MMP-2 and MMP-9 seems to be of limited value in predicting the survival of patients with TCC. Present results demonstrated MMp-2 and MMp-9 expressions were predictive factors of survival using the log-rank test ($p = 10^{-6}$, 10^{-6}, respectively), also these markers were independent predictive factors using multivariate analysis (Cox proportional hazard analysis). Present results also suggest that EGFr, MMP-2 and MMP-9 expressions are a potentially useful tool for the selection of the postoperative observation strategy and may be new valuable tumor markers for the screening, diagnosis and follow-up for patients with BTCC.

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