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THE SIGNIFICANCE OF EPIDERMAL GROWTH FACTOR RECEPTOR AND SURVIVING EXPRESSION IN BLADDER CANCER TISSUE AND URINE CYTOLOGY OF PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER
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ABSTRACT

***Objective*: To assess whether epidermal growth factor receptor (EGFR) and survivin immunostaining of tumour cells in urinary cytology and tissue of patients with bladder cancer has a prognostic significance.**
***Design*: Prospective study**
***Setting*: Department of Surgery (Division of Urology), Mubarak Al-Kabeer Teaching Hospital and Faculty of Medicine, Kuwait University, Kuwait**
***Subjects*: Urine cytology smears obtained prior to cystoscopy in patients with transitional cell carcinoma (TCC) of the bladder were immunostained for EGFR and survivin. Bladder cancer tissue resected at surgery was also immunostained for EGFR and survivin expression. Tissue expression of EGFR and survivin in TCC of the bladder was compared to their expression in urine cytology and relationship to tumour grade and stage.**
***Results*: 178 patients were studied (43 newly diagnosed bladder cancer, 58 with recurrent TCC and 77 in disease remission). Twenty five patients with normal urothelium served as controls. The mean sensitivity of urine cytology, tissue survivin immunohistochemistry (IHC) and tissue EGFR IHC was 30.5%, 62% and 59% respectively. The corresponding mean specificity was 95%, 79% and 38% respectively. For grades 1, 2 and 3 bladder tumors, tissue expression positivity for EGFR was 47.8%, 92.9%, 100% and for tissue survivin it was 27.8%, 18.2% and 33.3% respectively. For grades 1, 2 and 3 bladder tumors, urine expression positivity for EGFR was 35.7%, 40% and 67.7% and for urine survivin it was 8.3%, 42.9% and 33.3% respectively.**
***Conclusion*: Positive EGFR immunostaining of urine cytology specimen or tumour tissue increases with histological grade of TCC of the bladder. Survivin expression is less consistent in both urine cytology specimen and tissue samples. EGFR immunostaining may provide a useful tool in the grading of bladder TCC and aid in the selection of patients that may benefit from administration of EGFR inhibitors.**

INTRODUCTION

Currently, clinical stage (TNM category) is the most important prognostic factor influencing the outcome of treatment of transitional cell carcinoma (TCC) of the urinary bladder (1, 2). However, using the clinical stage to predict the outcome of patients with muscle-invasive bladder cancer treated with cystectomy is less accurate compared to pathological staging because of a tendency to under- or over-stage when clinical evaluation is used (3, 4). For muscle-invasive bladder tumours, cystectomy remains the best treatment option as 18-35% of patients with muscle-invasive disease will die from progressive

disease (4). Consequently, other prognostic markers are needed to improve the outcome of treatment of patients with muscle-invasive TCC of the bladder (1).

Advances in molecular biology in the past three decades have increased our understanding of the pathways that are altered in neoplastic cells. It has been suggested that protein expression profiling of TCC of the bladder offers an alternative means to distinguish aggressive tumour biology and may improve the accuracy of outcome prediction in patients treated with cystectomy (5). It has been shown that, structural and functional defects in key gate keepers of the cell cycle such as p53, p21, p27 cyclin E1 etc are common in human TCC of the bladder and have been associated with poor urologic outcomes (6-9). Many studies have reported that the accumulation of evidence on altered expression of many of these markers have added important prognostic information in patients with TCC of the bladder treated by radical cystectomy (5, 10, 11). Furthermore, the advent of immunohistochemical (IHC) techniques for the detection and quantitation of these markers has been shown to be useful in predicting tumour behaviour independent of classical prognostic factors (13). Two molecular markers are currently under assessment for use in addition to traditional staging techniques to assist in the staging of urothelial cancer. The 2 markers are epidermal growth factor receptor (EGFR) and survivin (14-22).

EGFR protein expression in bladder cancer is associated with increasing pathological grade and stage and higher rates of recurrence and disease progression [14, 15, 16]. Clinical studies have also shown both by molecular methods and IHC that there is a correlation between the levels of mutated p53 protein and muscle-invasive bladder cancer (13, 17, 18). Furthermore, EGFR is present in the urine of patients with bladder TCC.

Survivin is a member of the inhibitor of apoptosis protein family. It inhibits apoptosis via blocking caspase activation as do other inhibitors of apoptosis. It is also expressed in the G2 / M phase of the cell cycle and has been shown to be involved in the regulation of chromosome alignment and segregation (19). Expression of survivin in this phase may allow cells to overcome an apoptotic checkpoint, thus favoring aberrant progression through mitosis (20). Survivin has been shown to be highly expressed in many types of cancer cells including urothelial carcinoma (21). Its non-detection in non-proliferating normal adult tissues, makes it an attractive cancer therapeutic target (20). Even though some inconsistent results were obtained on the prognostic value of survivin protein or its messenger RNA (mRNA) level among many early studies in urothelial carcinoma, several recent studies using IHC or real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) have suggested that survivin expression may be a promising prognostic marker that correlates with unfavorable disease-specific survival and recurrence (22-24). In addition, survivin has also been proposed as a urine marker for early bladder cancer detection or detection of recurrence during follow-up (25, 26). This is because survivin is present in the urine of patients with urothelial cancer (25, 26).

In the present study, we correlated EGFR and survivin expression in bladder cancer tissues with other histopathological parameters. In addition, we correlated level of expression of EGFR and survivin in urine sediments obtained for urine cytology with the final histological grade of the resected tumours. We further determined the ability of tissue EGFR and survivin expression to diagnose bladder cancer in patients with newly diagnosed, recurrent TCC and patients with known TCC of bladder but in remission during surveillance.

PATIENTS & METHODS

Voided urine samples were obtained from patients with suspected TCC of the bladder (newly diagnosed patients), patients with known bladder TCC about to undergo scheduled surveillance check cystoscopy and patients with lower urinary symptoms requiring cystoscopy or elderly men with no LUTS requiring cystoscopy (normal urothelium). Urine specimens were sent for urine cytology and urine cytology smears (the slides) obtained were subsequently used for immunocytochemical assessment for the presence or absence of EGFR and survivin expression. All urine cytology and urine cytology EGFR and survivin expression were assessed by one experienced cytopathologist (KK).

All cystoscopic procedures and biopsies were carried out by an experienced urologist (EOK). Patients underwent transurethral resection of bladder tumour (TURBT) at diagnosis, or during surveillance cystoscopy if tumour was present. All tissues resected were subjected to routine histological procedures and sections stained with H&E for assessment of tumour grade and stage. These were determined by one experienced histopathologist (JTA). Tissue samples were further processed for IHC expression of EGFR and survivin. All IHC were graded positive (+ve) or negative (–ve) by one pathologist JTA.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of urine cytology, tissue EGFR and survivin expression on IHC in patients with newly diagnosed or recurrent TCC were determined and compared. Tumour grade was also compared with tissue EGFR and survivin expression. Similarly, tumour grade was also compared with EGFR and survivin expression in urine cytology smears. The final clinical stage of each tumour was determined in conjunction with findings at pre-operative IVU,

bone scan, CT scan and findings during cystoscopy including examination under anaesthesia (EUA). The final pathological grade was determined based on tissue resected at surgery using the WHO criteria of low grade (grades 1 and 2) and high grade (grade 3). We used clinical staging and not pathological stage because not all the patients had radical cystectomy.

Patients with known UTI, recent history of intravesical chemotherapy were excluded from the study as were patients with a final histological diagnosis of squamous cell carcinoma or adenocarcinoma. Patients with TCC of the renal pelvis and ureter were also excluded from this study. The study received local Ethical Committee approval and patients signed relevant informed consent forms.

Preparation of urine samples for cytopathological examination: Urine samples received (ranged from 5ml to 40ml) was concentrated by centrifugation (600g for 10 minutes). The supernatant was poured off and the cell pellet was vortexed to resuspend it. If the cell pellet was clear, 1 ml of PreservCyt solution was added, vigorously vortexed and four cytospins prepared on a ThermoShandon cytocentrifuge. If the cell pellet had a bloody appearance, 30 ml of CytoLyt solution was added to the cell pellet, centrifuged at 600g for 10 minutes, supernatant poured off and cell pellet vortexed prior to adding PreservCyt solution. Papanicolaou staining was done on one of the cytospins. The remaining cytospins were fixed in 95% ethanol and stored at -20ºc till staining was carried out. Immunostaining for EGFR and survivin was carried out on two of the cytospins and the remaining was stored at -20ºC as a reserve.

I*mmunostaining procedure for urine survivin and EGFR expression and scoring system:* The cytospins slides stored in the deep- freezer were again dipped in 95% alcohol for 30 minutes and then dehydrated. These were then quenched with 3% hydrogen peroxide to suppress endogenous peroxidase staining. After treatment with blocking reagent (Protein block, DAKO X0909) which prevents non-specific binding of subsequent reagents, samples were incubated with survivin (1:50) (Survivin D-8, sc 17779, Santacruz) and EGFR (1:50) overnight at 4ºC. Link antibody was added for 20 minutes followed by streptavidin-biotin (LSAB-DAKO) kit. Detection was achieved using a chromogen substrate solution diamino benzidine (DAB) [DAKO, S3000] and hydrogen peroxide. Slides were rinsed thrice with PBS after each step. The samples were counterstained with haematoxylin, dehydrated by passing through graded alcohols, cleared in xylene and mounted with DPX mountant. With each batch a positive control for survivin (normal stomach) and EGFR (placenta) was run. Pressure cooking in citrate buffer (0.01M, pH6.0), for eight minutes was done for the survivin control and enzymatic pepsin digestion for 10 minutes at 37 oC was done for EGFR control for antigen retrieval. Negative controls were run by omitting the primary antibody. Figures 1 and 3 show some positive survivin and EGFR expression respectively on urine cytology smears.

Figure 1
Survivin expression on urine cytology specimen (x 400)

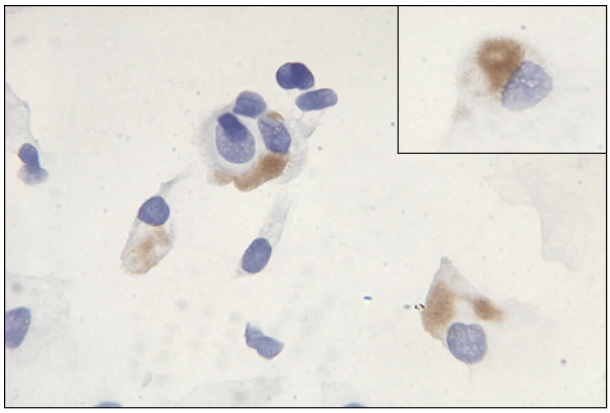


Figure 2
Survivin expression on resected bladder TCC tissue (x 1000)

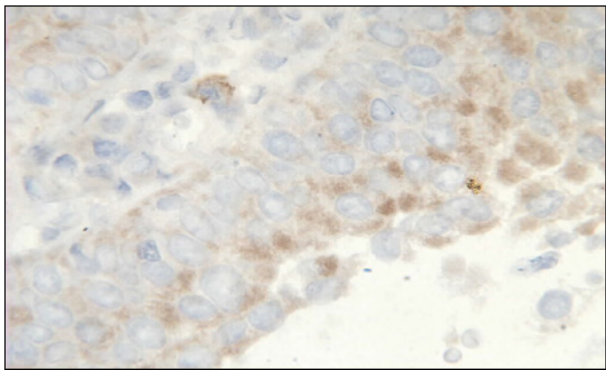


Figure 3a
EGFR expression on urine cytology specimen (x 200)

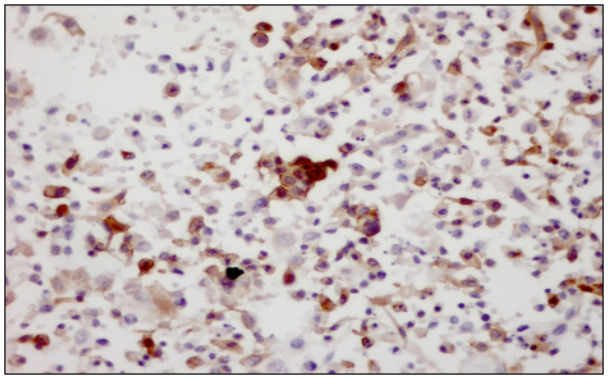
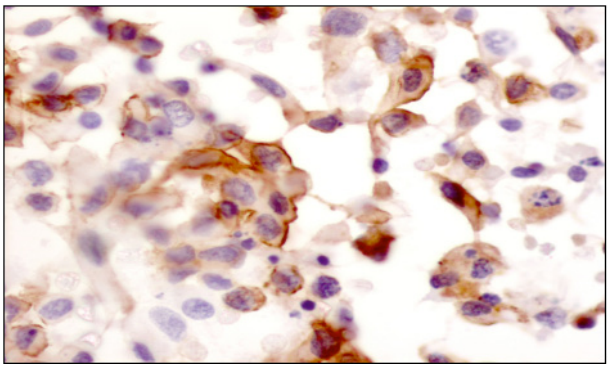
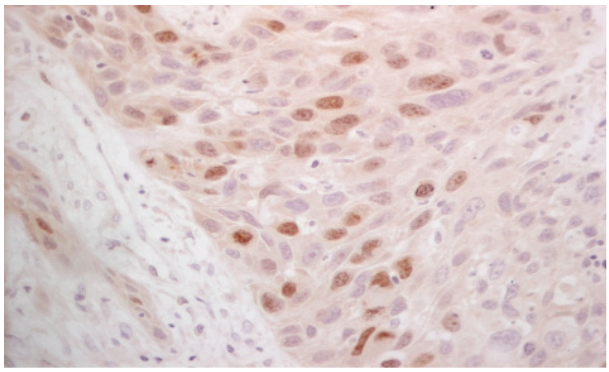


Figure 3b
EGFR expression on urine cytology specimen (x 400)



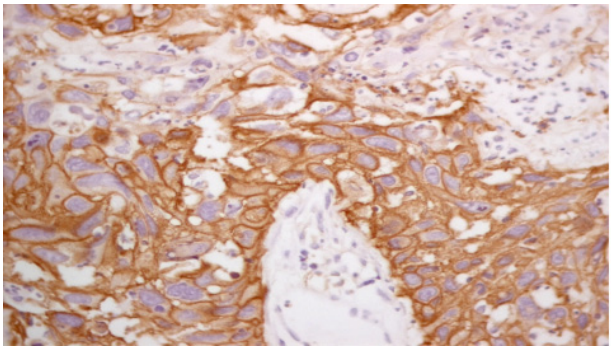
Survivin IHC: The technique used was similar to those described by Chen *et al* 2008 (19). Immunohistochemical staining for survivin (D-8, Santacruz Biotechnology, Santacruz, California, USA) was performed on formalin-fixed, paraffin-embedded, 5-μm tissue sections following appropriate antigen retrieval on the BondMax Autostainer (Vision BioSystems Inc, Norwell, Mass) using the Bond Polymer Define Secondary Detection System and using diaminobenzidine as the chromogen (Vision BioSystems). The selected survivin antibody was a mouse monoclonal antibody raised against aminoacids 1 to 142 of human survivin. An example of a positive tissue survivin expression is shown on Figure 4.

Figure 4
Positive tissue survivin expression in grade 3 TCC bladder. Survivin shows nuclear staining (x 250)



EGFR IHC: The technique used involved antigen retrieval by pepsin digestion and use of appropriate blocking solution. Primary antibody was followed by biotinylated link LSAB (Dako, Carpinteria, USA). DAB was used as chromogen, on IHC, EGFR positivity was as previously defined by Sriplakich *et al* 1999 (2). A tumour where ≥ 20% of the tumour cells, with the exception of those in the basal layer, showed membrane staining to the EGFR antibody in at least one of the examined fields (≥ 200) was defined as being EGFR-positive. In all other cases the tumour was considered EGFR-negative. When a tumour was EGFR-positive in only one or two fields, it was considered to be focally EGFR-positive. If stained areas were found in more than two fields, the tumour was considered to be diffusely EGFR-positive. An example of a positive tissue EGFR expression is shown on Figure 5.

Figure 5
Positive tissue EGFR expression in grade 3 TCC bladder. EGFR shows membrane staining (x 250)



Urine samples not analysable: Some urine specimens were not analysable for the following reasons:
for urine cytology, there was little or no cellular materials after cytospin to enable the cytopathologist make a definite diagnosis about the absence or presence of malignant cells. This was true for patients with small bladder cancer or those with very tiny recurrences: or EGFR and survivin immunostaining tests some results were equivocal, ie neither positive or negative or too few cellular material was present to make a definitive diagnosis. This was true for patients with very small bladder tumours or those with tiny recurrences.

We excluded these categories of urine samples from further analysis, based on the premise that we would consider a test +ve or –ve if we had enough cellular material to make a correct diagnosis.

Definitions:
In this study, the following definitions have been used;
Sensitivity = TP/TP + FN (i.e. tumour +ve),
Specificity = TN/TN +FP (i.e. tumour –ve),
PPV = TP/TP + FP
NPV = TN/TN + FN and
Diagnostic accuracy = TP+TN/n, where n = TP + FN + FP + TN.
Where T= true, N= negative, F= false, P= positive, PPV = positive predictive value and NPV = negative predictive value.
The sensitivity, specificity, PPV, NPV of urine cytology,

tumour tissue EGFR IHC staining, tumour tissue survivin IHC staining, urine sediment EGFR staining and urine sediment survivin staining were compared in patients with newly diagnosed TCC, patients with recurrent bladder TCC and the urothelium of those without tumour recurrence after cystoscopy and bladder biopsy. Factors affecting the sensitivity of the test (i.e. giving false +ve test results) were analysed. Furthermore, we analysed the effect of the final histological grade and stage of tumour on the sensitivity of each test.

STATISTICAL ANALYSIS

All data management and analysis were conducted using the SPSS software (SPSS Inc, Chicago, IL). The chi square ($\chi 2$) or Fischer's exact test was used to examine the association between categorical variables, and the normal Z test was used to assess the significant difference between two proportions. The t test was used to compare the means of two independent groups. A p value of <0.05 was considered statistically significant.

RESULTS

Table 1 shows the mean age (range), pathological stage and grade of the patients studied. The patients were fairly representative of each stage and grade of the disease. A total of 203 patients were studied (164 males, 39 females). The mean age of the patients was 52 ± 10.7 years. There were 43, 58 and 77 patients with newly diagnosed TCC, recurrent TCC and known TCC but in remission respectively, 25 patients who underwent cystoscopy but had no bladder outlet obstruction were used as controls. Bladder biopsies in the control group showed neither inflammation nor the presence of urothelial tumour. Patients with newly diagnosed bladder TCC had higher stage disease compared with those who had recurrent disease. Most of our patients presented with low grade disease as shown in Table 1.

Table 1
Pathological grade, stage and profile of patients with bladder cancer

Grade	Total number	Newly	Diagnosed**		Recurrent**		In	remission	Control	+Gross	Total
	of cases*	No	%	No	%	p**	No	%		No	%
1	55	23	53.5	32	55.2	0.09	45	58.4		100	56.2
2	36	13	30.2	23	39.7	0.06	27	35.1		63	35.4
3	10	7	16.3	3	5.1	0.001	5	6.5 a		15	8.4
Total (n)	101	43	100	58	100		77	100	25	203	100
Stage	Total	Newly	Diagnosed**		Recurrent**		In	remission	Control		
		No	%	No	%	p**	No	%			
CIS	6	4	9.3	2	3.4	0.001	9	11.7		15	8.4
					Superficial						
pTa	6	4	9.3	2	3.4	0.001	22	28.6		28	15.7
pT1	54	17	39.5	37	63.9	0.1	42	54.5		96	53.9
					Invasive						
pT2	16	7	16.3	9	15.5	0.1	4	5.1		20	11.2
pT3	9	5	11.5	4	6.9	0.07	0	0		9	5.1
pT4	10	6	14.0	4	6.9	0.001	0	0		10	5.6
Total	101	43	100	58	100		77	100	25	203	100
Mean age (range) yrs		53 (16-77)	57 (23-71)		59 (16-76)	55 (20-70)	52 (16-77)				

*The numbers shown in this column of the table represent total number of patients recruited and with tissue available for histological analysis following bladder biopsy or TURBT.
** Fischer's exact test

+Gross total includes patients with disease, those in remission and control subjects.
'a' patients with grade 3 or stage >PT1 urothelial tumour considered for cystectomy except it was very focal in nature or patient was subjected to partial cystectomy.

Table 2

Performance characteristics of urine cytology, survivin and EGFR immunohistochemistry on tissues obtained from patients with bladder cancer, but who had no tumour recurrence at follow up cystoscopy + bladder biopsy

Types of patients	Remission (Tissue Diagnosis)*		
Test	Urine cytology (n=56) N (%)	EGFR on IHC BT (n=21) N (%)	Survivin on IHC BT (n=28) N (%)
False positive	3 (5.4)	13 (62)	6 (21)
True negative	53 (95)	12 (38)	22 (79)

n: represented the number of tests performed for each marker out of 77 patients in remission. Patients whose urine samples were not used for UC, EGFR or survivin IHC had very few cells in the urine – under the category of patients with TCC bladder in remission.
IHC BT = immunohistochemistry on bladder tissue
*Diagnosis based on histology of tissue biopsy.

Table 3

Performance characteristics of urine cytology, survivin and EGFR immunohistochemistry on bladder tissue specimens in patients with bladder cancer.

Tests	Test	N D TCC	R C T TCC	Test	N D TCC	R C T TCC	Test	N D TCC	R C T TCC
1.Sensitivity	UC	28.0%	33.0%	Survivin IHC BT	63.0%	61.0%	EGFR IHC BT	57.0%	61.0%
2.Specificity	UC	95.0%	95.0%	Survivin IHC BT	79.0%	79.0%	EGFRIHC BT	38.0%	38.0%
3. PPV	UC	84.9%	86.8%	Survivin IHC BT	75.0%	72.3%	EGFR IHC BT	48.0%	49.6%
4. NPV	UC	84.1%	58.6%	Survivin IHC BT	68.1%	67.0%	EGFR IHC BT	46.9%	49.4%
5.Diagnostic Accuracy	UC	62.0%	64.0%	Survivin IHC BT	71.0%	70.0%	EGFR IHC BT	47.5%	48.5%

UC = urine cytology,
ND TCC = newly diagnosed TCC
RCT TCC = recurrent TCC
IHC BT = immunohistochemistry on bladder tissue

Table 4

Comparison of EGFR and survivin expression on urine cytology specimen with corresponding histological grades of bladder tumour resected

Histological Grade	UCS EGFR +ve	UCS Survivin +ve	p
1	(n = 5/14) 35.7% 1,6	(n = 1/12) 8.3% 1,7	1p = 0.001
2	(n = 2/5) 40% 2	(n = 3/7) 42.9% 2	2p = 0.15
3	(n = 4/6) 67.7% 3,6	(n = 2/6) 33.3% 3,7	3p = 0.2
No recurrence	(n = 5/30) 16.7% 4	4p = 0.001	
Normal Urothelium (Control)	(n = 15/27) 18.5% 5	5p = 0.04	

Fischer's Exact test = 1-5p.
'Z' test for trend = 6,7p. 6p = 0.01, 7p = 0.001
UCS = urine cytology specimen

Table 5
Comparison of tissue EGFR and survivin expression with corresponding histological grade of bladder tumour resected

Histological Grade	Tissue EGFR +ve	Tissue Survivin +ve	p
1	(n = 11/23) 47.8% 1,4	(n = 5/18) 27.8% 1,5	1p = 0.2
2	(n = 13/14) 92.9% 2	(n = 2/11) 18.2% 2	2p = 0.001
3	(n = 9/9) 100% 3,4	(n = 1/3) 33.3% 3,5	3p = 0.001
No recurrence	(n = 13/28) 46.4% 6	(n = 6/28) 21.4% 6	6p = 0.06
Normal Urothelium (Control)	(n = 8/25) 32% 7	(n = 6/25) 24% 7	7p = 0.3

Fischer's Exact test (1,2,3,6,7p)
'Z' test for trend: 4p = 0.01, 5p = 0.2

The sensitivity of urine cytology, tissue survivin expression (+ve or –ve) and EGFR tissue expression (+ve or –ve) are shown in Tables 2 and 3. Similarly, the specificity, PPV, NPV of each test for all categories of patients and controls are shown in Tables 2 and 3.

Table 4 and Figures 1 and 3 show the positivity of EGFR and survivin expression on urine cytology smears compared with the final tissue diagnosis and tumour grade. The EGFR expression increased from 35.7% to 67.7% for grade 1 to grade 3 tumours. There was inconsistent expression of survivin on urine cytology smears. EGFR expression both on tissue and urine cytology smears increased with the tumour grade. The normal urothelium also showed minor EGFR and survivin expression in about equal proportion for each marker as shown in Table 4.
Table 5 and Figures 2, 4 and 5 show the relationship between tumour grade on positivity of tissue EGFR and Survivin expression. There was progressive increase in expression of tissue EGFR from 47.8% in well differentiated cancers to 100% in poorly differentiated TCC. The tissue expression of survivin was inconsistent.

DISCUSSION

TCC of the bladder is currently the commonest urological neoplasm in the Middle East for two major reasons, the high prevalence of tobacco smoking and the high prevalence of Schistosoma haematobium infection in many parts of the Middle East. From tTable 1, our patients also presented at an earlier age compared to those reported in the literature and more than 80% were current or ex-smokers. Superficial bladder tumours are amenable to TURBT in addition to intravesical chemotherapy. However, for patients with invasive disease, treatment is by cystectomy. It has been reported that a significant proportion of patients treated by cystectomy will experience relapse of their disease owing to unrecognized and untreated nodal or distant metastases at the time of surgery. Hence, the need for a search for better prognostic markers, to enable stratification of patients into those in whom adjuvant therapy will be required to prevent recurrence or to prolong survival (5, 27).

In the search for novel therapeutic approaches, members of the EGFR/erbB family have emerged as attractive objects for targeted therapy. Thus adjuvant chemotherapy with the monoclonal antibody Trastuzumab to Her-2 (of the EGFR family) has become a standard regimen in the treatment of Her-2-over-expressing breast carcinomas (27). The small molecule EGFR-inhibitor gefitinib (ZD 1839, Iressa) was approved for the treatment of non-small cell lung cancer by the FDA in 2003 (28) and is currently being tested in clinical trials of patients suffering from colorectal cancer and various other malignancies (27). EGFR overexpression has been widely reported in urothelial carcinomas of the bladder. However, the presence of EGFR has shown significant variation between 23% and 95% among different tumours, and statistical analyses have also resulted in varying conclusions regarding its prognostic significance (27, 29, 30). The frequent expression on EGFR in urothelial carcinomas with squamous and/or glandular differentiation is of particular interest, because metaplastic features have been shown to; correlate with both high tumour stage and grade (31), and also correlate with a decreased response to conventional radiotherapy (32) and chemotherapy (33). The fact that both EGFR expression and metaplastic differentiation correlate with high tumour stage and grade suggests that EGFR expression may be involved in the progression of this particular histological subtype (27).

In this study, we also found that tissue overexpression of EGFR in particular correlates well with tumour grade with the expression being positive in 47.8% of grade 1 tumours compared to 100% in grade 3 tumours. However, EGFR expression was also found to be positive in the urothelium of 46.4% of patients with no tumour recurrence on bladder

biopsy. This indicates that, EGFR tissue expression will be of prognostic significance only when tissue histology is positive for tumour. For the purpose of diagnosis, the sensitivity of tissue EGFR positivity is higher than urine cytology indicating that in equivocal or tiny tissue biopsies, the expression of EGFR may assist in clarifying potential neoplasm from benign tissue. Leibl *et al* 2008 (27)showed that TCC with metaplastic and glandular differentiation showed strong EGFR expression indicating that strong EGFR expression of TCC tumours should be considered in patient selection for neoadjuvant therapy as well as interpretation of clinical trial targeting EGFR. This is because, TCC with metaplastic and glandular differentiation have been shown to be more resistant to chemotherapy and radiotherapy (27).

In this study, unlike EGFR expression in tissue and urine cytology smears which showed a consistent pattern with tumour grade and stage, survivin expression did not correlate well with stage or grade. This may be because of the type of survivin antibody used by us. This is because several different survivin antibodies have been used in previous studies and nuclear as well as cytoplasmic staining have been reported (21, 22). Survivin is known to be associated with mitotic apparatus, centromere of chromosomes, and mitocondria, and different splice variants were also reported to have unique nuclear or mitochondrial localisations (34). As polyclonal antibodies tend to generate non specific background staining, in this study we used a monoclonal antibody raised against the full-length human survivin protein to avoid this potential pitfall. The survivin IHC pattern we observed was predominantly nuclear, with focal weak cytoplasmic staining in some cases that we did not find to correlate with tumour grade as shown in Table 4. A similar observation was made by Chen *et al* 2008 (19). This clean staining pattern suggests that some of the previously reported cytoplasmic staining with polyclonal anti-survivin antibodies might have been background staining, and data correlating such cytoplasmic staining to prognosis should be interpreted with caution (19). In this study, we failed to confirm previous findings by Chen *et al* (19) who showed that survivin scores correlated better and had higher predictive value than histology for high grade recurrent tumours as only 33.3% of our high grade tumours stained positively with survivin.

The role of EGFR inhibitors in the adjuvant setting following cystectomy for muscle invasive urothelial cancer has started to receive attention in recent times (35). This is because survival remains poor for this category of patients. Pruthi *et al* (35) showed that the EGFR inhibitor erlotinib when administered in the neoadjuvant setting can have beneficial effects in terms of surgical pathology and short-term clinical outcomes in patients undergoing radical cystectomy for invasive bladder cancer. Thus, the search must continue for a better understanding of the molecular biology and genetics of cancer which will hopefully lead to the identification of a host of candidate targets for more specific anticancer treatment (36). Against this background, the advent of novel therapeutic agents that specifically target these genetic alterations may help to revolutionise the treatment of cancers and result in improvement of survival with minimal toxicity (35, 36). Patients with urothelial tumours with positive EGFR expression will be expected to have better response to EGFR inhibitors like erlotinib than patients with negative or low expression (35).

A potential limitation of our study is the reliability of IHC techniques. IHC is semiquantitative and dependent on a range of variables such as the choice of antibody used, antibody concentration, fixation techniques, variability in the interpretation and stratification criteria, inconsistency in specimen handling and other technical procedures (5). These problems with 'visual' IHC can be minimized by the use of tissue microarrays using an automated scoring system based on brightfield microscopy imaging coupled with advanced color detection software (5). The adoption of these modern techniques has been shown to result in better standardization and greater reproducibility of IHC (34, 37, 38).

In conclusion, the present study suggests that EGFR immunostaining in tissue and urine cytology specimen, but not survivin possibly has prognostic and predictive value in the management of patients with TCC of the bladder. Visual IHC results are difficult to compare and the evaluation of the results of immunostaining differ greatly between centers. Standardisation of these procedures using microarray techniques may improve the results and possibly aid in the incorporation of IHC into the overall management of patients with urothelial cancer.

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