Expression of Epidermal Growth Factor Receptor and Transforming Growth Factor Alpha in Cancer Bladder: Schistosomal and Non-Schistosomal

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Abstract
Introduction: Overexpression of epidermal growth factor receptor (EGFR) has been described in several solid tumors including bladder cancer. Transforming growth factor alpha (TGFα) is frequently deregulated in neoplastic cells and plays a role in the development of bladder cancer. TGFα-EGFR ligand–receptor combination constitutes an important event in multistep tumorigenesis. Methods: This study was done on 30 bladder biopsies from patients with urothelial carcinoma, 15 with squamous cell carcinoma, 10 with cystitis and 5 normal control bladder specimens. All were immunochemically stained with EGFR and TGFα antibodies. Results: EGFR and TGFα were over-expressed in higher grades and late stages of bladder cancer. Moreover, they show higher expression in squamous cell carcinoma compared to urothelial carcinoma and in schistosomal associated lesions than in non-schistosomal associated lesions. Conclusion: EGFR and TGFα could be used as prognostic predictors in early stage and grade of bladder cancer cases, especially those with schistosomal association. In addition they can help in selecting patients who can get benefit from anti-EGFR molecular targeted therapy.
has been reported to be involved in the pathological processes of several cancers through interaction with other ligands like transforming growth factor alpha (TGFα) [8]. EGFR plays critical roles in cell growth, differentiation, motility, and survival [9]. It was reported to be overexpressed in a variety of solid malignancies including UC [10]. Several investigators have suggested that expression of EGFR could be utilized for molecular targeted therapy in urinary bladder cancer [11–13].

TGFα is a mitogenic polypeptide that has a wide range of biological activities including self-renewing epithelia; it acts as a paracrine growth factor through its interaction with EGFR [14]. The ability of TGFα to contribute to transformation and oncogenesis occurs through the activation of EGFR [15]. Overexpression of TGFα appears to be an event in bladder cancer that occurs more frequently in schistosomal bladder cancer and SCC, and may play an important role in their development. These observations may provide insight into treatment guided by molecular changes [16].

The present study was conducted to evaluate immunohistochemical expression of EGFR and TGFα in urothelial cells of chronic cystitis and urinary bladder cancer with or without schistosomal affection.

### Materials and Methods

#### Specimens

A total of 55 archival urinary bladder blocks from Pathology Department of Theodor Bilharz Research Institute were included in this study; obtained either as transurethral resections or radical cystectomy specimens. They consisted of 10 cases of cystitis, 30 UC and 15 SCC. They belong to 47 males and 8 females (mean age 55.41 ± 12.73 years, range 27–74 years). In addition, 5 normal bladder specimens were obtained from patients subjected to prostatectomy after taking their consent, and served as controls (mean age 66.00 ± 5.38 years, range 57–71 years).

#### Histopathological Study

Five micron serial sections were stained with hematoxylin & eosin. The 60 biopsies were categorized into the following groups: Group 1: Control (n = 5); Group 2: Cystitis/schistosomal (n = 5) and non-schistosomal (n = 5); Group 3: UC/schistosomal (n = 17) and non-schistosomal (n = 13); Group 4: SCC/schistosomal (n = 10) and non-schistosomal (n = 5).

#### Histological Grading

Urinary bladder tumors were histologically divided into 3 grades (I–III).

#### Pathological staging

Staging of bladder tumors followed WHO classification [17]. Tumors of grade I are considered of low grade, while those in grades II and III are of high grade. Tumors of pathological stage T1 are considered superficial and that > T1 are muscle invasive.

Schistosomal infection was diagnosed upon presence of Schistosoma ova in tissue samples.

#### Immunohistochemical technique

Formalin-fixed paraffin embedded sections (5μm in thickness) were cut. Sections were incubated in oven at 60 °C overnight, deparaffinization and rehydration were done. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide. Antigen retrieval was performed by microwaving the sections in citrate buffer, pH 6.0. Sections were incubated overnight at 4 °C in humid chamber with the primary antibodies: EGFR monoclonal antibody (Dako, Code no. Ab1006, Glostrup, Denmark) and TGFα polyclonal antibody (Abcam, Ab 9578, Cambridge, Massachusetts, USA), at an optimal dilution of 1:50 for both, with application of ultravision detection system HRP Polymer. The antigen was localized by the addition of DAB (3, 3’ Diaminobenzidine) substrate chromogen solution. Finally, slides were counterstained with hematoxylin, dehydrated in alcohol and mounted.

For each setting, negative controls were carried out in which phosphate buffered saline was used instead of the primary antibody. Skin biopsies were used as positive controls for both antibodies.

#### Interpretation of Immunostaining

All immunostained slides were assessed and scored. The sections were examined by using light microscope (Zeiss, Germany). For EGFR, immunopositivity was indicated by brownish cytoplasmic or membranous staining in the urothelial cells while TGFα alpha positivity was detected as brownish cytoplasmic staining in

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**Table 1.** Grades and stages of studied malignant bladder lesions

| Category       | UC               | SCC               | Total              |
|----------------|------------------|-------------------|--------------------|
| Grade          | n (%)            | n (%)             | n (%)              |
| I              | 11 (36.7)        | 4 (26.7)          | 15 (33.3)          |
| II             | 12 (40)          | 6 (40)            | 18 (40)            |
| III            | 7 (23.3)         | 5 (33.3)          | 12 (26.7)          |
| Pathological stage |                  |                   |                    |
| T1             | 15 (50)          | 5 (33.3)          | 20 (44.4)          |
| T2             | 11 (36.7)        | 5 (33.3)          | 16 (35.6)          |
| T3             | 4 (13.3)         | 5 (33.3)          | 9 (20)             |
| Total          | 30               | 15                | 45                 |

| Category       | Non-schistosomal | Schistosomal      | Total               |
|----------------|------------------|-------------------|---------------------|
| Grade          | n (%)            | n (%)             | n (%)               |
| I              | 8 (44.4)         | 7 (26)            | 15 (33.3)           |
| II+III         | 10 (55.6)        | 20 (74)           | 30 (66.7)           |
| Pathological stage |                  |                   |                     |
| Superficial (T1) | 11 (61.1)       | 9 (33.3)          | 20 (44.4)           |
| Invasive (T2+T3)| 7 (38.9)         | 18 (66.7)         | 25 (55.6)           |
| Total          | 18               | 27                | 45                  |

UC = Urothelial carcinoma; SCC = squamous cell carcinoma.

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**Table 2.** Effect of Schistosomal association on tumor grade and stage

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urothelial cells. The percentage of positive cells was evaluated in 10 microscopic fields at power \( \times 400 \) and the mean value was obtained.

The percentage of stained cells in each section was estimated on a scale of 0–3 (extent of expression), 0 (no positive cells), 1+ (positivity in 1–20% of cells), 2+ (positivity in 21–50% of cells), and 3+ (positivity in > 51% of cells). Immunostaining intensity of EGFR and TGF\(_\alpha\) were scored into mild, moderate and marked.

**Statistical Analysis**

SPSS for Windows, version 20 was used for statistical analysis (IBM Corporation, Armonk, New York, USA). Means of different groups were compared using one-way ANOVA. The correlation between EGFR and TGF\(_\alpha\) scores of immunoreactivity and histopathological stage, grade and schistosomal association was assessed by Spearman correlation coefficient. A p value < 0.05 was considered significant.

**Results**

**Histopathology**

Among studied urothelial tumors (Group 3), UC was diagnosed in 30/45 (66.7%) and SCC in 15/45 (33.3%). Urothelial tumors were found to be 15 (33.3%), 18 (40%) and 12 (26.7%) in grades I, II, and III respectively. Twenty (44.4%), 16 (35.6%) and 9 (20%) were in stage T1, T2, and T3 respectively (table 1). Schistosomiasis was diagnosed in 32/55 (58.2%) of all studied lesions. It was diagnosed in 50% of chronic cystitis cases and in 60% of malignant cases (56.6% of UC cases and 66.6% of SCC cases). In Schistosomal associated tumors, 20/27 (74.1%) were muscle invasive (T2+T3), and 18/27 (66.7%) were of high grade (grade II or III) versus 55.6% and 38.9% of non-Schistosomal associated tumors (table 2).

**EGFR Immunoexpression**

All control and chronic non schistosomal cystitis cases were negative for EGFR staining. Positivity of EGFR was expressed in urothelial cells as brownish cytoplasmic and/or membranous staining. Only one case of chronic schistosomal cystitis (1/5) was positive for EGFR with weak expression (score 1+) showing mild staining intensity (table 3, Histogram 1 and fig. 1A).

**Table 3. EGFR expression in studied groups**

| Studied groups (n) | Positive cases n (%) | % of positive cells (Mean ± SD) | Extent of EGFR immunoreactivity | Score 1+, n (%) | Score 2+, n (%) | Score 3+, n (%) |
|-------------------|----------------------|--------------------------------|--------------------------------|-----------------|-----------------|-----------------|
| Control (5)       | 0                    | 0                              | –                              | –               | –               | –               |
| Chronic cystitis (10) | 1 (10)               | 2.00 ± 6.32                    | 1 (100)                        | 0               | 0               | –               |
| Non-schistosomal cystitis (5) | 0            | 4.00 ± 8.94                    | –                              | 1 (100)         | –               | –               |
| Schistosomal cystitis (5) | 39 (86.7)*          | 43.51 ± 23.54*                 | 0                              | 21 (53.8)       | 18 (46.2)       | –               |
| Carcinomas (45)   | 15 (83.3)            | 39.44 ± 24.54                  | 0                              | 11 (73.3)       | 4 (26.7)        | –               |
| Schistosomal carcinomas (27) | 24 (89)          | 46.22 ± 22.92                  | 0                              | 10 (41.7)       | 14 (58.3)       | –               |

EGFR = Epidermal growth factor receptor; *Significant difference compared to control and chronic cystitis (p < 0.01).

**Table 4. EGFR expression in malignant lesions**

| Histological diagnosis (n) | Positive cases n (%) | % of positive cells (Mean ± SD) | Extent of EGFR immunoreactivity | Score 1+, n (%) | Score 2+, n (%) | Score 3+, n (%) |
|---------------------------|----------------------|--------------------------------|--------------------------------|-----------------|-----------------|-----------------|
| UC (30)                   | 24 (80)              | 41.10 ± 26.51                  | 0                              | 13 (54.2)       | 11 (45.8)       | –               |
| Non-Schistosomal (13)     | 10 (77)              | 37.30 ± 28.32                  | 0                              | 7 (70)          | 3 (30)          | –               |
| Schistosomal (17)         | 14 (82.4)            | 44 ± 25.53                     | 0                              | 6 (43)          | 8 (57)          | –               |
| SCC (15)                  | 15 (100)             | 48.33 ± 15.77                  | 0                              | 8 (53.3)        | 7 (46.7)        | –               |
| Non-Schistosomal (5)      | 5 (100)              | 45.00 ± 10.00                  | 0                              | 4 (80)          | 1 (20)          | –               |
| Schistosomal (10)         | 10 (100)             | 50 ± 18.25                     | 0                              | 4 (40)          | 6 (60)          | –               |

EGFR = Epidermal growth factor receptor; UC = urothelial carcinoma; SCC = squamous cell carcinoma.
(mean% of positive cells) rose significantly in malignant cases versus cases of chronic cystitis (p < 0.01) (table 3, histogram 1). All cases of SCC were positive for EGFR versus 80% of UC cases without significant difference in its extent of expression or staining intensity between both tumor types (table 4, fig. 1B–D).

**Fig. 1.** Immunohistochemistry for EGFR in urinary bladder sections expressed as brown cytoplasmic and membranous staining of urothelial cells (A) Schistosomal cystitis (score 1+, × 200), (B) TCC Grade I (score 2+, × 400), (C) TCC Grade II (score 3+, × 400), (D) SCC with schistosomiasis (arrowed) Grade II (score 3+, × 400). EGFR = epidermal growth factor receptor; TCC = transitional cell carcinoma; SCC = squamous cell carcinoma.

**Histogram. 1.** Intensity of EGFR expression in studied groups
Schistosomal association with bladder cancer increased extent of EGFR expression (score 3+) as well as its staining intensity compared to non Schistosomal cancer cases in both cancer types (table 3, 4 and histogram 1).

As regards the studied grades and stages of bladder cancer; EGFR positivity, extent of expression as well as moderate staining intensity were found to be increased in grade 3 and stage 3 than in lower ones (table 5, histogram 2 and fig.1B–D).

**Fig. 2.** Immunohistochemistry for TGFα in urinary bladder sections expressed as brown cytoplasmic staining of urothelial cells. (A) Schistosomal cystitis (score 1+, × 200). (B) Papillary TCC Grade II (score 3+, × 400). (C) TCC Grade III (score 3+, × 400). (D) SCC Grade II (score 3+, × 200). TGF = transforming growth factor alpha; TCC = transitional cell carcinoma; SCC = squamous cell carcinoma.

**Histogram. 2.** Intensity of EGFR in different grades and stages of positive malignant cases. G = Grade; T = Stage.
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TFG alpha immunoexpression

TGFα positive staining was indicated by brown cytoplasmic staining in urothelial cells. Forty percent of control cases showed TGFα immunopositivity (2 out of 5 cases), all were in score 1+, with sequential increase to 70% in chronic cystitis and 91.2% in bladder cancer cases. Similarly, the extent of TGFα expression (mean % of positive cells) increased significantly in bladder cancer over chronic cystitis and control cases (p < 0.01). Schistosomal association with cystitis and bladder
Histogram 3. Intensity of TGF-α expression in studied groups.

Histogram 4. Intensity of TGF-α in different grades and stages of positive malignant cases. G = Grade; T = Stage.

Table 8. TGF-α expression in malignant grades and stages

| Histological diagnosis (n) | Positive cases n (%) | Extent of TGF-α immunoreactivity | % of positive cells (Mean ± SD) | Score 1+, n (%) | Score 2+, n (%) | Score 3+, n (%) |
|---------------------------|-----------------------|---------------------------------|--------------------------------|----------------|----------------|----------------|
| Grade                     |                       |                                 |                                |                |                |                |
| Grade I (15)              | 14 (93.3)             | 50.53 ± 22.95                   | 3 (21.5)                       | 5 (35.7)       | 6 (42.8)       |
| Grade II (18)             | 16 (88.8)             | 50.00 ± 24.25                   | 0                              | 7 (43.7)       | 9 (56.3)       |
| Grade III (12)            | 11 (91.6)             | 53.25 ± 27.49                   | 0                              | 2 (18.2)       | 9 (81.8)       |
| Stage                     |                       |                                 |                                |                |                |                |
| Stage 1 (20)              | 17 (85)               | 47.40 ± 26.20                   | 3 (17.6)                       | 6 (35.3)       | 8 (47)         |
| Stage 2 (16)              | 16 (100)              | 54.31 ± 5.49                    | 0                              | 7 (43.7)       | 9 (56.3)       |
| Stage 3 (9)               | 8 (88.8)              | 53.3 ± 8.33                     | 0                              | 1 (12.5)       | 7 (87.5)       |
cancer increased TGFα positivity, and extent of expression in score 3+ compared to non Schistosomal cases. All positive cases of cystitis either schistosomal or non-schistosomal showed mild staining intensity, while in carcinoma (schistosomal and non-schistosomal) they were mainly of moderate and marked intensity (table 6 and histogram 3).

In SCC TGFα positivity was 100%, while being 86.7% in UC cases. Extent of TGFα expression (mean % of positive cells) was significantly higher in SCC than in UC (p < 0.01). In addition schistosomal association increased TGFα positivity and extent of expression in score 3+ compared to non Schistosomal cases in both SCC and UC (table7 and fig. 2).

No detectable changes in TGFα positivity, or staining intensity among different grades or stages of studied tumors, but extent of expression of score 3+ increased in higher stages and grades than in lower ones (table 8 and histogram 4).

Spearman’s correlation coefficient (r) calculation revealed significant positive correlation between schistosomal association and EGFR positivity (r = 0.628, p = 0.001), EGFR score (r = 0.619, p = 0.001), EGFR staining intensity (r = 0.279, p = 0.01), TGFα positivity (r = 0.403, p = 0.001), TGFα staining intensity (r = 0.184, p = 0.01), and TGFα score (r = 0.305, p = 0.05). EGFR score correlated significantly with tumor grade (r = 0.352, p = 0.01) and stage (r = 0.389, p = 0.01). Also TGF score correlated significantly with tumor grade (r = 0.312, p = 0.05) and stage (r = 0.263, p = 0.05).

Discussion

Different studies have established that bladder cancer is the most common cancer in Egypt and most cases were linked with Schistosomiasis [7]. EGFR has been associated with the genesis of bladder tumors [18]. Over-expression of EGFR leads to uncontrolled cell proliferation. It also results in increased angiogenesis and reduced apoptosis, processes necessary for continuing malignant growth [19]. TGFα contributes to transformation and oncogenesis through the activation of EGFR [15].

In our study we detected EGFR immunostaining as membrano-cytoplasmic staining of urothelial cells; this finding is in accordance with results observed by Khaled et al. [13] and Abdulamir et al. [20].

Our 5 control cases were negative for EGFR; this finding is consistent with results of Khaled et al. [13]. Cheng et al. [21] stated that 40–60% of human bladder tumors overexpress EGFR mRNA and protein, while Naik et al. [7] reported positive EGFR expression in only 23% of their malignant cases. In our study 86.7% of malignant lesions were positive for EGFR; 83% of non schistosomal and 89% of schistosomal carcinoma. Abdulamir et al. [20] recorded EGFR overexpression in 58% and 84% of non schistosomal and schistosomal bladder tumors, and Khaled et al. [13] reported its expression in 66% of schistosomal bladder cancer.

In current study schistosomal association increased percentage of positive cases for EGFR expression in both cystitis and malignant cases. This finding is consistent with data reported by Abdulamir et al. [20] who concluded that schistosomal cystitis might act as an intermediate stage between normal and tumor tissues indicating the danger of the long-lasting inflammation of the bladder.

For histological types of bladder cancer, EGFR immunopositivity was found in all SCC cases compared to 80% of UC cases; this is in accordance with Guo et al. [22] and Ibrahim et al. [23] who reported that SCC of the urinary bladder, which is frequently related to schistosomal etiology and an advanced stage, expresses more intensely enhanced levels of EGFR.

Significant association reported in current study between increased EGFR expression with high grade and late stage of cancer bladder is in agreement with Colquhoun et al. [24] and Khaled at al. [13], suggesting that EGFR signaling may play a role in tumor progression [25]. However, Popove et al. [18] demonstrated that EGFR expression had no additional prognostic value over clinical stage, grade or cell proliferation. A study of Memon et al. [26] provided an explanation for this controversial data since they found that in some cases EGFR expression alone shows no correlation with survival, yet a high expression of EGFR together with increased Her3 and Her4 correlate with a better survival, denoting the existence of a synergistic effect between EGFR and other Erb family members mainly Her3/4.

In the current study, TGFα immunoreactivity was detected in 40% of control cases, this agrees with that reported by Tungekar et al. [27]. TGFα positivity increased to be 70% in chronic cystitis and 91.2% in bladder cancer cases matching with data reported by Mellow et al. [28].

Schistosomal association increased the percentage of positive cases for TGFα expression in both cystitis and malignant cases. This finding is consistent with data reported by Swellam et al. [16] who concluded that TGFα appears more frequently in schistosomal bladder cancer.
and SCC, suggesting that it may play an important role in their development. These observations may provide insight into treatment guided by molecular changes.

In our study, TGFα immunopositivity was found in all SCC cases compared to 86.7% of UC cases; this is in accordance with results reported by Swellam et al. [16] and Tungekar et al. [27].

Swellam et al. [16] found an association between late stages, and high histological grades with TGFα positivity. In the current work, TGFα also expressed with higher scores in high grades and late tumor stage.

In conclusion, EGFR and TGFα overexpression were found to be more frequent in bladder malignant lesions and stronger in schistosomal versus non-schistosomal cases. EGFR and TGFα overexpression correlated with higher tumor grade and stage, thus can be used as predictors for prognosis.

The current treatment of advanced bladder cancer relies mainly on traditional cytotoxic agents. High expression of biologic markers such as EGFR and TGFα can provide a preclinical proof of concept that anti-EGFR therapy can be used to target bladder tumors expressing these markers.

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