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Evaluation of epidermal growth factor receptor, transforming growth factor alpha, epidermal growth factor and c-*erbB2* in the progression of invasive bladder cancer

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Abstract *Introduction*: Determination of the risk of invasive bladder tumors progressing is still imprecise due to the heterogeneous biological behavior of this neoplasm. The goals of this study were to evaluate the patterns of expression of the epidermal growth factor (EGF) system in invasive bladder cancer and to assess its prognostic value.
Methods: This immunohistochemical study was performed using fresh frozen tumor samples and a panel of monoclonal antibodies on a series of 43 invasive bladder cancers treated by cystectomy.
Results: EGF was detected in 45% of the tumors and did not correlate with survival from bladder cancer. Transforming growth factor alpha (TGFα) was expressed by 60% of the tumors and correlated strongly with death from bladder cancer. Epidermal growth factor receptor (EGF-R) expression was seen in 86% of cases and had no prognostic significance. c-*erbB2* was expressed in 50% of cases and was inversely related to a poor prognosis. When EGF and TGFα were both expressed, there was little or no expression of c-*erbB2*.
Conclusion: The accumulation of several growth factors and the relevant receptor are necessary for the progression of invasive bladder cancers. They could be used as indicators of tumor aggressiveness.

Key words Growth factors · Bladder cancer · Invasive tumors · Prognosis · EGF · Receptor

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Introduction

Bladder cancer is a common malignancy with a heterogeneous biological behavior. There are different growth potentials even within the same groups of tumors as defined by classical prognostic factors [6]. The determination of the risk for progression is still imprecise, despite the development of new prognostic markers. Altered expression of the retinoblastoma gene (*Rb* gene) has been shown to be a prognostic indicator in patients with invasive bladder cancer [3]. E-cadherin is expected to be a prognostic marker, and, for patients with invasive tumors, conservation of normal E-cadherin staining gives a relatively good prognosis [1]. Overexpression of *p53* seems to predict a poor outcome in muscle-invasive tumors, with no independent prognostic value over clinical stage [16]. DNA ploidy [35] may be useful for prognostic determination since aneuploidy is strongly linked to poor clinical outcome.

Growth factors represent a wide group of low molecular weight polypeptides (1.5–50 kDa) which are closely linked to several cellular mechanisms including proliferation, motility and transformation. All responsive targets have cell membrane receptors that specifically bind the appropriate ligand. There are a growing number of reports concerning the significance of activated proto-oncogenes and abnormal growth factor production in the induction of tumor cell growth and its association with an aggressive phenotype in many tumor groups. One of the best-characterized growth

factor pathways is the epidermal growth factor (EGF) system, which together with other factors controls some biological mechanisms [7]. EGF is a 53-amino-acid single-chain protein with a molecular weight of 6.45 kDa. It is produced by several cell types in a precursor form and has both growth stimulatory and inhibitory effects on normal and tumoral cells of many types. It is linked to the epidermal growth factor receptor (EGF-R), which is its physiologic receptor and is found to be excreted in high concentrations in a biologically active form in the urine.

Transforming growth factor alpha (TGFα) belongs to a family of heat- and acid-stable growth factors [28]. It is a phylogenetic and functional relative of EGF but with only limited structural homology. It is a ligand for EGF-R as well. Increased amounts are found in the urine of patients with disseminated cancers [33]. It seems likely that it is derived from the tumor itself since increased expression of TGFα mRNA has been identified in a variety of tumors including renal cell carcinoma, breast cancer and squamous cell carcinoma.

EGF-R is a 170-kDa transmembrane glycoprotein which is widely distributed in normal tissues, and is the product of the c-*erbB1* gene, which is located on chromosome 7. It is a tyrosine kinase receptor with autophosphorylation of tyrosine residues after binding to its ligands [8]. Recent studies have indicated that EGF-R and its ligands are implicated in several mechanisms in the oncogenesis of certain tumors of epithelial origin (breast, bladder) and its expression is a marker of poor prognosis [12, 21, 30].

Another oncogene, *neu* (c-*erbB2*), which is closely related to *erbB1*, is located on chromosome 17q21 [19]. This proto-oncogene encodes a transmembrane 185-kDa glycoprotein which has 50% structural homology with the EGF-R amino acid sequence. It belongs to the tyrosine kinase receptor family and its putative ligand, heregulin, has only recently been identified [10]. Amplification of the c-*erbB2* gene and overexpression of its protein product have been reported mainly in adenocarcinomas, and in breast cancer is associated with a poor prognosis [4].

To date there has been a limited number of studies evaluating EGF-R [14, 20, 24, 26, 27, 31, 32], EGF [13], and c-*erbB2* [2, 17, 22, 34, 37, 38] in transitional cell carcinoma and to our knowledge no reports regarding the prognostic significance of TGFα in this tumor. The concurrent expression of these has also not been reported. The goals of this study were to evaluate the patterns of immunostaining for EGF-R, EGF, TGFα and c-*erbB2* in invasive bladder cancer and to assess their prognostic value alone and in association with each other. We also studied the relationship between EGF-R and c-*erbB2* expression.

Material and methods

Study population

All patients included in this study had invasive bladder cancer (stage T1 or higher) and underwent radical cystectomy or cystoprostatectomy (42 patients) or partial cystectomy (1 patient) at Harper Hospital, Detroit, MI, between 1983 and 1992. The criteria for inclusion were the availability of fresh frozen tumor samples in the frozen tumor bank and clinical follow-up information. Patient characteristics including age at cystectomy, sex, treatment and outcome data were obtained from chart review and physician contact. Slides from the radical cystectomy specimens were reviewed and tumors assessed for histologic type, histologic grade (WHO) [23] and pathologic stage (1987 UICC) [11].

Immunohistochemical procedures

All specimens were obtained at the time of radical cystectomy. Each sample was snap frozen and stored at −70 °C. A control H&E-stained section at the beginning and end of sectioning was performed in order to confirm the presence of tumor within the frozen tissue sample. Frozen tissue sections cut at 5 μm were immersed in cold acetone for 10 min and then rehydrated. Slides were then incubated for 20 min with the primary antibody (60 min with EGF). Commercially available monoclonal antibodies against EGF, TGFα, EGF-R (Ab1, Ab2 and Ab1, respectively, from Oncogene Science Inc.) and c-*erbB2* (Triton Biosciences, Inc.) were used. Dilutions were 1/20, 1/200, 1/200 and 1/2000, respectively. Positive and negative controls were used for each antibody. Slides were washed in modified phosphate-buffered saline (MPBS) and then incubated for 10 min with the secondary antibody at 1/200 dilution (biotinylated rabbit anti-mouse immunoglobins). Slides were washed again in MPBS. Finally, an avidin and biotinylated peroxidase complex (ABC Vectastain, Vector Laboratories) was incubated for 10 min. Samples were then washed in double-distilled water, counterstained and mounted in Immuno-mount (Shandon) under cover-slips.

A sample was considered negative when immunostaining of the tumor cells was the same as the negative control. Positive samples were assessed for staining intensity (zero, weak, moderate, strong) and extent (focal, diffuse) independently by two examiners (D.J.G., V.R.). In cases of disagreement, a third examination under a double-headed microscope was carried out and a final assessment agreed upon. For purposes of data analysis, positivity of a sample was defined by the presence of at least focal staining of moderate or strong intensity. Lesser degrees of reactivity were combined with those showing a complete absence of immunoreactivity. All slides were reviewed without knowledge of outcome data.

Statistical analysis

Distribution characteristics (means ± SD) for quantitative variables such as age and frequency tables for quantitative demographic and clinical data were generated for all variables. All study variables were tested by chi-square (two-tailed test) and Fisher's exact test for small samples (one-tailed test) for significant bivariate associations. Each variable was tested for prognostic significance for mortality due to bladder cancer using Kaplan-Meier survival curves and the log-rank test. A multivariate analysis for demographic and clinical variables was tested using the Cox stepwise regression model. All statistical analyses were performed using the SAS statistical software program. Results were considered significant at $P = 0.05$.

Table 1 Clinicopathologic data and immunohistochemical results

Case	Age (years)	Sex	Race	Stage	Grade	ECF-R	EGF	TGFα	c-*erbB2*	FU (months)	Outcome
1	63	M	C	T1N0	3	+	+	+	+	38	A + W
2	72	M	C	T1N0	3	+	+	−	+	69	A + W
3	84	M	C	T1N0	2	+	−	−	+	15	A + W
4	51	F	C	T2N0	3	−	+	+	−	84	A + W
5	61	M	B	T2N0	3	+	−	−	+	19	A + W
6	67	M	C	T2N0	3	+	−	−	−	23	DUC
7	62	M	C	T3aN0	3	−	−	−	−	17	A + W
8	63	M	B	T3aN0	3	+	−	−	+	72	A + W
9	73	M	C	T3aN0	3	+	−	−	−	47	A + W
10	75	M	C	T3aN0	2	+	−	−	+	64	DUC
11	80	M	B	T3aN0	2	+	−	−	+	19	DUC
12	34	F	C	T3aN1	3	+	−	+	−	19	DOD
13	55	M	C	T3aN2	3	−	+	−	+	44	A + W
14	58	M	B	T3bN0	3	+	+	+	+	43	DUC
15	61	M	C	T3bN0	3	+	+	+	+	34	DOD
16	63	F	B	T3bN0	2	+	−	−	+	20	DUC
17	65	F	C	T3bN0	3	+	+	+	−	15	DOD
18	72	M	C	T3bN0	3	+	−	−	−	68	A + W
19	74	F	C	T3bN0	3	−	+	+	−	14	DOD
20	75	M	C	T3bN0	3	+	+	+	+	14	DOD
21	77	M	C	T3bN0	3	+	+	+	+	18	DOD
22	78	M	C	T3bN0	3	+	+	+	+	10	DOD
23	81	M	C	T3bN0	3	+	+	+	−	30	A + W
24	72	M	C	T3bN1	3	+	−	+	−	15	A + W
25	74	F	C	T3bN1	3	+	−	−	−	18	A + W
26	80	M	C	T3bN1	3	+	+	+	−	41	DOD
27	45	M	C	T3bN2	3	+	+	+	+	13	DOD
28	75	M	B	T3bN2	3	N/A	N/A	N/A	N/A	10	DOD
29	51	M	C	T4N0	3	+	−	+	−	25	A + W
30	54	M	C	T4N0	3	+	−	−	+	08	A + W
31	63	F	C	T4N0	3	+	−	+	−	23	DOD
32	75	M	C	T4N0	3	+	−	+	+	17	DUC
33	76	M	C	T4N0	3	+	+	+	−	07	DOD
34	76	M	C	T4N0	3	+	+	+	−	08	DOD
35	66	F	B	T4N1	2	+	−	−	+	20	A + W
36	67	F	C	T4N1	3	+	+	+	−	14	DOD
37	34	M	C	T4N2	3	+	−	+	+	15	DOD
38	57	F	C	T4N2	3	−	+	+	−	26	DOD
39	62	M	C	T4N2	3	+	+	+	−	15	DOD
40	63	M	C	T4N2	3	+	−	−	−	06	DOD
41	63	M	C	T4N2	3	+	−	+	−	09	DOD
42	67	F	C	T4N2	3	−	−	+	−	12	DOD
43	67	F	B	T4N2	3	+	−	−	−	07	DOD

(Abbreviations – *M* male, *F* female, *C* Caucassian, *B* African-American, *N/A* not available, *FU* follow-up, *A + W* alive and well, *DUC* dead from unrelated cause, *DOD* dead from bladder cancer)

Results

The patient population consisted of 31 males and 12 females (Table 1); age ranged from 34 to 84 years (mean 66 years, median 67 years). Pathologic stage was pT1 in 3 cases; pT2, 3 cases; pT3a, 7 cases; pT3b, 15 cases; and pT4, 15 cases. Lymph node metastases were documented in 16 patients (37%). Two patients with stage T1 disease underwent radical cystectomy for extensive, multifocal disease and for associated diffuse transitional cell carcinoma in situ which had failed bacille Calmette-Guérin (BCG) therapy, respectively. The third patient with T1 disease had tumor involving a diverticulum and was treated by partial cystectomy. Of the 43 patients, 22 (51%) died of bladder cancer. Follow-up for those not dead of cancer ranged from 8 to 84 months (median 23 months, mean 35 months). There were 41 transitional cell carcinomas, 1 squamous cell carcinoma and 1 small cell carcinoma. The histologic grade was 2 of 3 in 5 cases and 3 of 3 in the remaining 38 cases. Tumor stage was a highly significant predictor of outcome ($P = 0.0002$; log-rank test). The presence of lymph node metastases was also a poor prognostic indicator ($P < 0.02$; log-rank test). Histologic grade was not a significant predictor of outcome ($P = 0.066$, log-rank test).

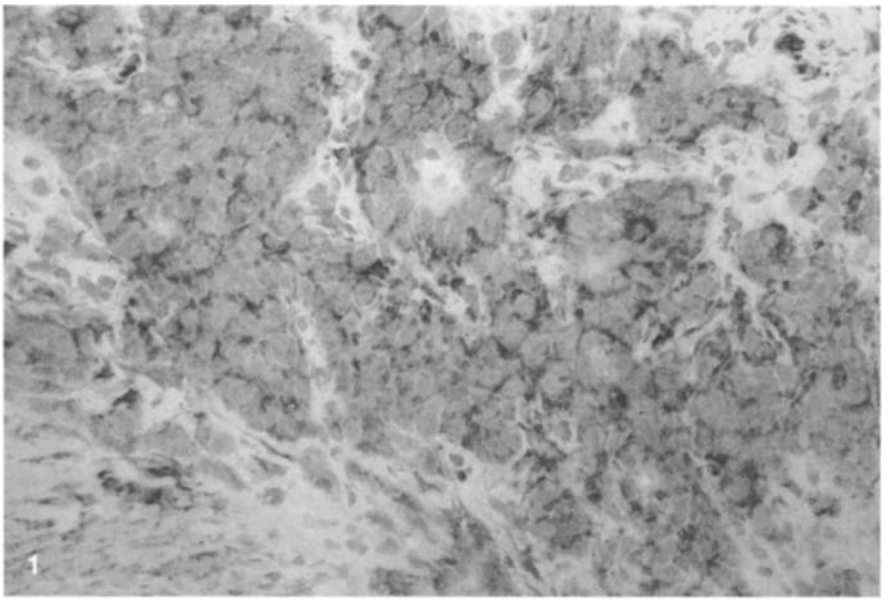

Fig. 1 Invasive high-grade transitional cell carcinoma showing strong expression of TGFα. Immunohistochemical stain, ×190

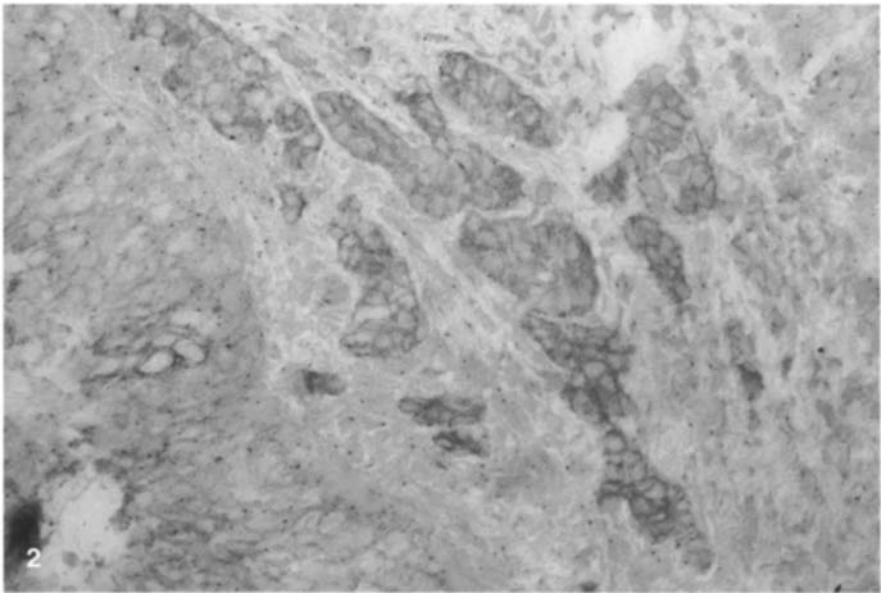

Fig. 2 Strong expression of EGF in an invasive high-grade transitional cell carcinoma. Immunohistochemical stain, ×190

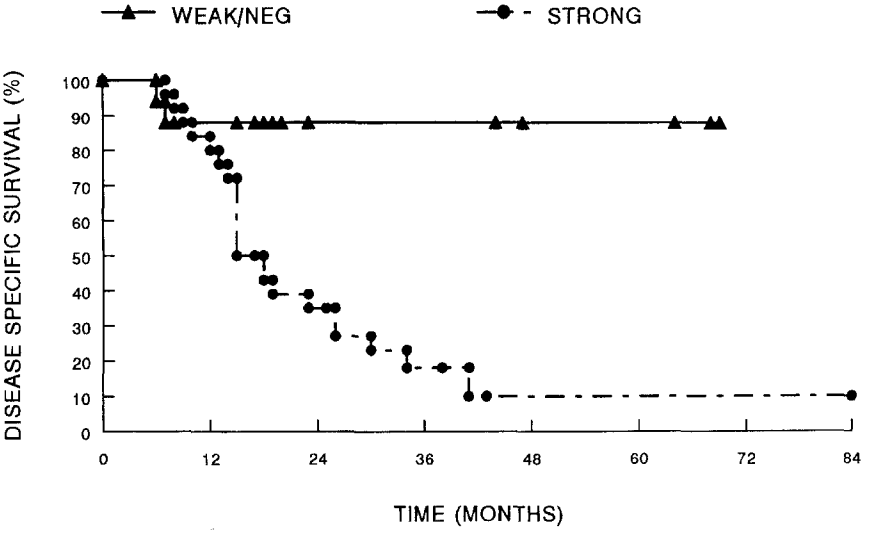

Fig. 3 Kaplan-Meier survival curve of disease-specific survival by TGFα expression. $P = 0.0009$, log-rank test

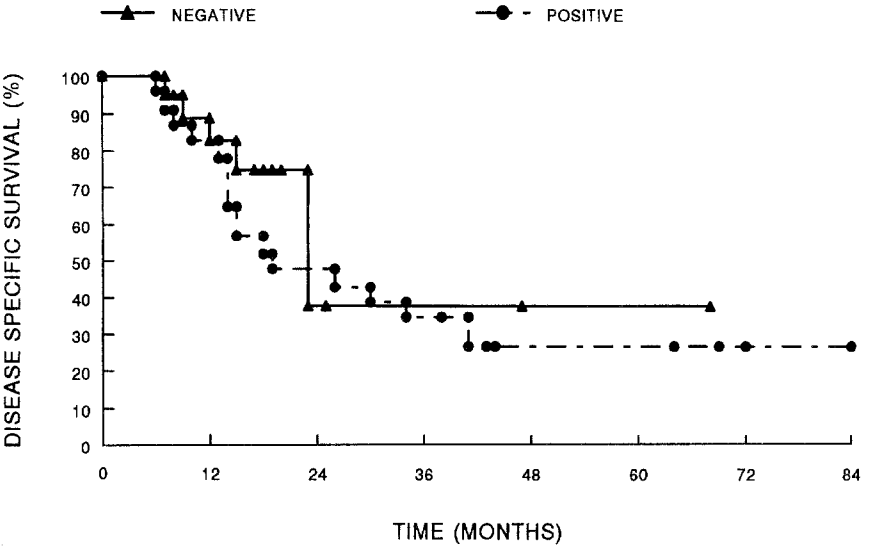

Fig. 4 Kaplan-Meier survival curve of disease-specific survival by EGF expression. $P > 0.05$, log-rank test

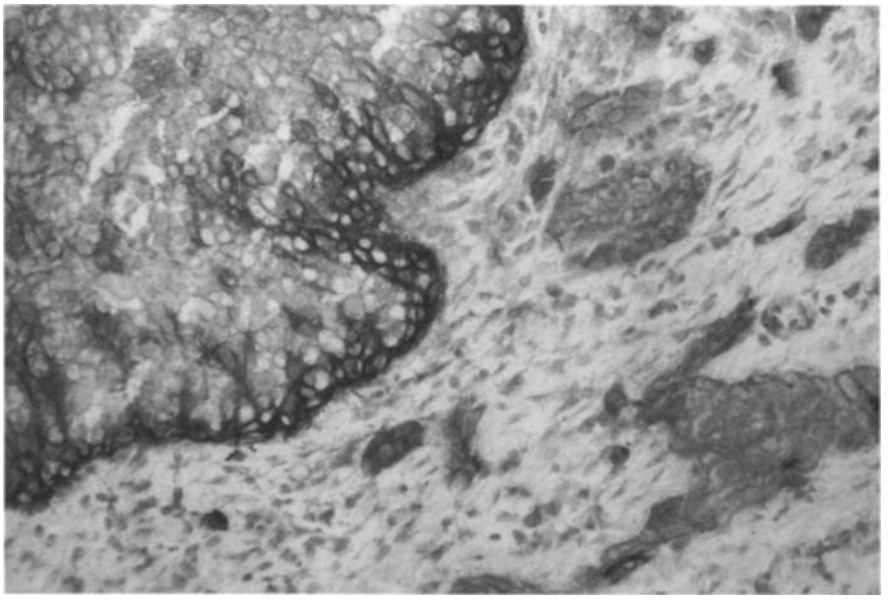

Fig. 5 Invasive high-grade transitional cell carcinoma with strong immunoreactivity for EGF-R. Immunohistochemical stain, $\times 190$

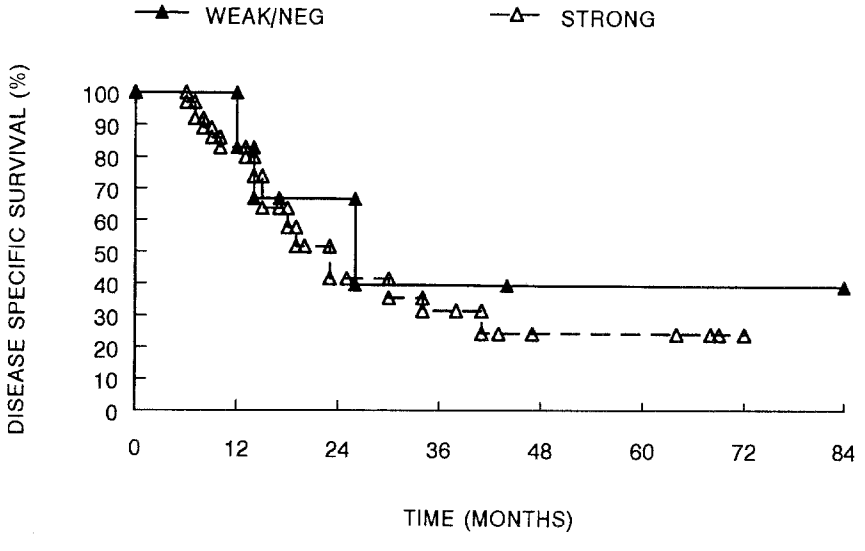

Fig. 6 Kaplan-Meier survival curve of disease-specific survival by EGF-R expression. $P > 0.05$, log-rank test

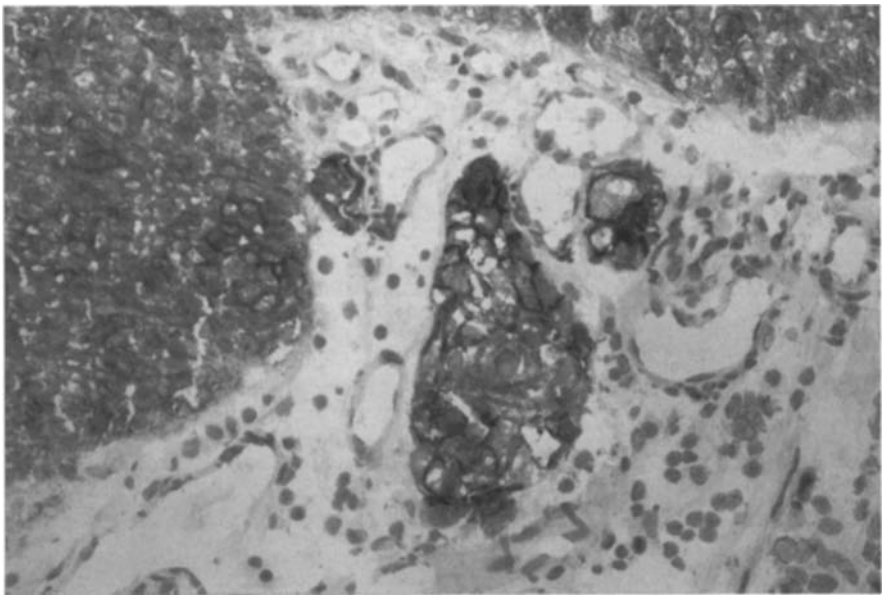

Fig. 7 Strong positive immunoreactivity for c-*erbB-2* in a case of invasive transitional cell carcinoma. Immunohistochemical stain ×190

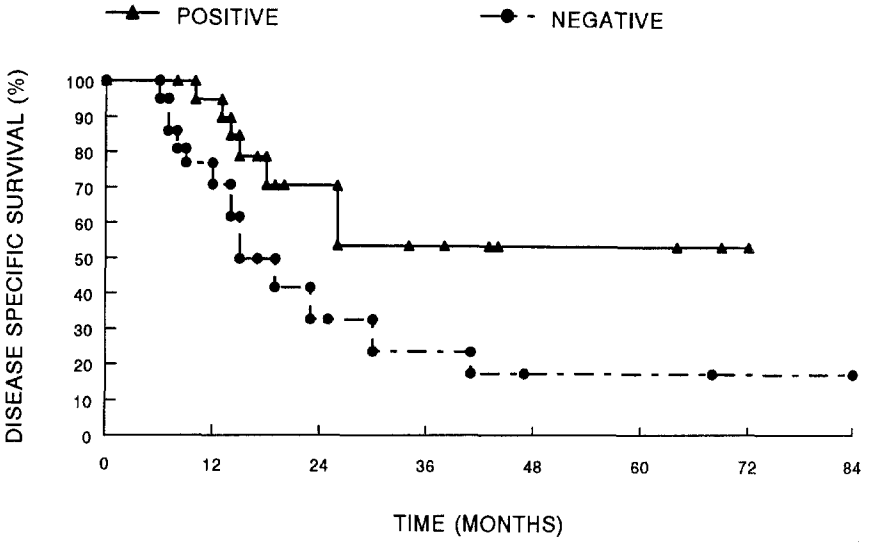

Fig. 8 Kaplan-Meier survival curve of disease-specific survival by c-*erbB-2* expression. $P = 0.04$, log-rank test

Immunohistochemical results were available for 42 of 43 cases. In one case no tumor remained in the sections stained. The number of tumor cells stained for EGF, TGFα and EGF-R varied among individual cases and intra-tumoral staining heterogeneity was often present. TGFα and EGF were expressed in tumor cells by 60% (25/42) and 45% (19/42) of the samples, respectively. TGFα and EGF staining was observed in both the cytoplasm and on the membranes, but neither were necessarily present in the same areas (Figs. 1, 2) of any given sample. TGFα was expressed within stromal tissue as well while EGF staining was constantly weak as compared to TGFα staining. There was a significant association between TGFα expression and tumor stage ($P < 0.05$, chi-square) but not with grade ($P > 0.05$; chi-square) and a strong association with decreased survival ($P = 0.0009$; log-rank test; Fig. 3). EGF expression correlated weakly with grade ($P = 0.05$; chi-square) but not with stage ($P = 0.09$; chi-square) or survival ($P = 0.10$; log-rank test; Fig. 4). The majority of tumors expressed EGF-R (36/42, 86%) with strong membrane reactivity (Fig. 5), while in most of them there was an increasing EGF-R expression from basal cells to superficial ones. There was no correlation between EGF-R expression and grade ($P > 0.05$; chi-square), stage ($P > 0.05$; chi-square) or survival ($P > 0.05$; log-rank test; Fig. 6). The oncogene c-*erbB2* was expressed in 21 cases (50%) (Fig. 7). The staining was strongly and homogeneously expressed on the cell membrane. Interestingly, this was inversely correlated with decreased survival ($P = 0.04$; log-rank test; Fig. 8). There was a weak association between c-*erbB2*

expression and grade ($P = 0.04$; chi-square) but not with stage ($P > 0.05$; chi-square). No relationship was observed between c-*erbB2* and EGF-R expression. Of 19 EGF-positive samples, 10 were c-erbB2 positive (52.6%) and of 25 TGFα-positive samples, 10 were c-erbB2 positive (40%). Coexpression of EGF-R and both its ligands was statistically correlated with poor outcome ($P < 0.03$; log-rank test).

In the Cox stepwise regression model, pathologic stage was the most significant predictor of death from bladder cancer ($P = 0.002$). TGFα approached statistical significance ($P = 0.09$). In this analysis the Cox regression model is limited by the small number of patients.

Discussion

Increased levels of EGF-R have been found in several cancer systems. Several groups have reported on the expression of this receptor in bladder cancers. In most studies the authors have reported good correlations between EGF-R expression and tumor stage, histologic grade and size, with overexpression most often seen in deeply invasive and high-grade bladder cancers [13–15, 26, 27, 39]. Expression in muscle-invasive tumors has been found in up to 100% of cases [20]. EGF-R expression has also been found to correlate with higher proliferation rates in bladder carcinoma [25, 32]. In our panel of invasive cancers, there was overexpression of EGF-R in most cases, in accordance with previous studies. In prognostic studies, the presence of EGF-R in non-muscle-invasive tumors has been found to be predictive for time of first recurrence and for future progression [26]. It has also been associated with cancer death, but in these studies no significance was demonstrated when the data from patients with muscle-invasive tumors were analyzed separately [26, 27]. We also found no correlation between the expression of this receptor and clinical outcome, suggesting that EGF-R on its own has no prognostic value for locally advanced bladder tumors. The high EGF-R expression rate (86%) may explain why this receptor failed to demonstrate any prognostic value in this group of patients.

EGF is distributed in various tissues, but it has been suggested that the tubular and ductal epithelium of the kidney is the primary site of EGF synthesis [13]. There are only limited data available on EGF expression in bladder cancer and, to our knowledge, there are no prognostic data available for EGF expression in invasive bladder cancers. In our analysis, EGF expression could be demonstrated in 45% of invasive bladder carcinomas but was not a significant prognostic indicator.

TGFα has a multiplicity of biological effects on a variety of mesenchymal and epithelial cells, alone or in combination with other growth factors [5]. Gleave et al. [9] have demonstrated that TGFα and basic fibroblast growth factor (bFGF) expression can be induced by both autocrine and paracrine mechanisms in a bladder cancer cell line. They also showed the effects of TGFα can be inhibited by anti-EGF-R antibodies. There has been no previous prognostic analysis of TGFα expression in invasive bladder cancers. In our series, expression of TGFα was seen in 60% of cases and correlated significantly with outcome. In the multivariate analysis this approached but was not independent of stage, however, the number of cases studied is too few for firm conclusions to be reached. Several recent studies have considered EGF-R and its ligands together as potential prognosticators in cancer [36]. If the coexpression of TGFα, EGF and high levels of EGF-R are needed to contribute to tumor development in vivo, then these events should be coselected in human malignancies. Our study has demonstrated that the coexpression of EGF-R and both its ligands (TGFα, EGF) was significantly related to a poor prognosis. This correlation is of considerable interest since the expression of a receptor has been closely linked to the ligand binding (in experiments of cellular growth).

Amplification and overexpression of c-*erbB2* has been reported in a number of different human tumor systems. In bladder tumors, preliminary reports have been contradictory. Wood [37] found that DNA amplification occurred infrequently in bladder cancers but c-*erbB2* mRNA was overexpressed in 36% of tumors. This report thus indicates that overexpression of c-*erbB2* in the absence of gene amplification is the primary mechanism in bladder cancer as well. Swanson [34] did not find any relationship between c-*erbB2* immunoreactivity and tumor stage (50% in superficial and 50% in invasive tumors), clinical outcome or DNA content. In contrast, Wright [38] found a weak correlation between c-*erbB2* status and tumor stage and Moriyama [22] reported that c-*erbB2* was more frequently expressed in high-grade and high-stage tumors. Coombs [2] and Lipponen [16] also indicated that there was a relationship between grade and gene amplification or immunohistochemical status of the protein. The latter study, utilizing paraffin-embedded specimens from deep and superficial transitional cell carcinoma (TCC), found that moderate and strong expression of c-*erbB2* was related to a poor prognosis [17]. One explanation for the conflicting results is perhaps the significant variability in the sensitivity and specificity of the antibodies used in different studies [29].

In this series of predominantly muscle-invasive tumors, expression of c-*erbB2* was inversely related to prognosis in contradiction to previous reports. This inverse relationship was also found by Nguyen [27], who, similarly to us, studied patients ($n = 85$) with invasive TCC treated by cystectomy. In that report

c-*erbB2* positivity was seen in 29% of cases (compared to 50% in our series). The loss of c-*erbB2* expression could represent a major change in the cell immunophenotyping that could be linked in part to the modifications of the tumor behavior towards aggressiveness. We also noted that when EGF and/or TGFα were expressed, there was often no expression of c-*erbB2*; this could indicate that downregulation of c-*erbB2* is caused by EGF and/or TGFα. Marth [18] found that TGFα and EGF reduced the measurable amount of c-*erbB2* protein in ovarian and mammary cell lines, perhaps because of inducing internalization and rapid degradation of c-*erbB2* (thus not recognized by the antibody). The mechanism for this relationship, however, requires further study. In our study there was no correlation between expression of the c-*erbB2* product and EGF-R, a finding which is consistent with one previous report [22].

In summary, the accumulation and interactions of several growth factors appears to be important for the progression of invasive cancer of the urinary bladder. These may be attributed to genetic changes including activation of oncogenes, inactivation and deletion of anti-oncogenes and transcriptional regulatory sequences. The altered expression of these genes might occur independently, but may be co-selected for, as a result of the proliferative advantage conferred. This study shows that c-*erbB2* and the EGF system may be involved in the biology of invasive bladder cancers.

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