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THE IMMUNOHISTOCHEMICAL DETECTION OF CRIPTO-1 IN BENIGN AND MALIGNANT HUMAN BLADDER

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SUMMARY

The recently identified epidermal growth factor-related peptide cripto-1 has been previously implicated in the development of the malignant phenotype. The identification of gene products that can act as prognostic markers in bladder cancer would be of value in determining the management of this heterogeneous group of patients. This study examines cripto-1 expression in benign and malignant bladder using immunohistochemical techniques. The expression of cripto-1 protein in benign and malignant bladder was examined in 45 bladder tumours (Ta/T1 $n=26$, T2 $n=5$, T3/T4 $n=14$) and six benign controls. All 45 tumours showed positive cytoplasmic staining for cripto-1, including areas of carcinoma *in situ*. None of the six benign controls showed any evidence of positive cripto-1 staining. Twenty-three (60 per cent) bladder tumours had areas of papillary tumour that showed strong positive staining for cripto-1 as opposed to six (29 per cent) sections of histologically normal urothelium adjacent to tumour ($P<0{\cdot}05$). There was no association between cripto-1 staining and tumour grade, stage, or clinical outcome. Cripto-1 protein appears to be specifically expressed in malignant and benign adjacent urothelium of patients with bladder cancer. Its clinical significance, however, remains to be determined. © **1998 John Wiley & Sons, Ltd.**

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KEY WORDS—**cripto; bladder cancer; immunohistochemistry**

INTRODUCTION

Abnormal expression of growth factors and their corresponding receptors have been implicated in the formation and progression of a variety of malignancies.[1–3] The CRIPTO-1 gene was first identified and cloned from an undifferentiated human embryonal carcinoma cell line, NTERA2 clone D1.[4] The CRIPTO-1 gene encodes for a 188-amino acid glycoprotein containing a region of 37 amino acids that has structural homology, with epidermal growth factor (EGF), but lacks a hydrophobic signal peptide and transmembrane domain. This 37-amino acid region contains six cysteine residues that can form three intramolecular disulphide bonds and thus confers partial secondary structural homology with other members of the EGF/TGF-α family, such as transforming growth factor-α (TGF-α) and amphiregulin.[5] The human CRIPTO-1 gene is 4·8 kb and is located on chromosome 3p 21. It is organized into six exons, with exon 4 containing the entire EGF/TGF-α-like segment. The receptor for cripto-1 has not yet been identified, but it may be an additional *erb*B-related receptor. Cripto-1 has been shown to function as a growth factor through an EGF receptor-independent pathway.[6]

The CRIPTO-1 gene is capable of *in vitro* transformation of both mouse NIH3T3 fibroblasts[4] and a normal mouse mammary epithelial cell line, NOG-8, as assessed by the ability to form clones in soft agar,[7] thus demonstrating that cripto-1 can function as a dominantly transforming oncogene. This was demonstrated in the NOG-8 cells by introduction of an expression vector plasmid containing the human CRIPTO-1 cDNA.[7] However, this transformed cell line was unable to form tumours when injected into nude mice, suggesting that expression of cripto-1 may not be sufficient to produce the malignant phenotype *in vivo*.

Further studies have shown elevated expression of cripto-1 mRNA and protein in human primary breast, gastric, colon, and pancreatic cancers.[7–11] In addition, inhibition of cripto-1 expression in human colon cancer cells by antisense RNA and oligodeoxynucleotides led to the formation of significantly smaller tumours in nude mice compared with controls, implicating cripto-1 as an important growth modulator in the control of colon cancer.[12] In view of the possible role of cripto-1 in the development of cancer this study examines its expression in human bladder cancer using immunohistochemical techniques.

PATIENTS AND METHODS

Patients

Forty-five patients with histologically diagnosed transitional cell carcinoma of the bladder and six benign

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controls were studied. Twenty-six superficial (Ta or T1) and 19 muscle invasive tumours (T2–T4) were stained for cripto-1. Five patients with invasive tumours and one patient with superficial disease had additional areas of carcinoma *in situ* (CIS). Of the six benign controls, two were from patients with cystitis, two were from patients with benign prostatic hyperplasia (BPH) but histologically normal bladders, and two were from patients investigated for lower urinary tract symptoms with normal bladder on histopathological evaluation. Of the 26 patients with superficial tumour (Ta/T1), 18 (69 per cent) were male with a median age of 66 years (range 46–81 years) and eight (31 per cent) were female with a median age of 71 years (range 54–87 years). Of the 19 patients with muscle invasive disease (T2: $n=5$; T3: $n=10$; T4: $n=4$), 13 (68 per cent) were male with a median age of 69 years (range 51–80 years) and six were female with a median age of 71 years (range 58–82 years). The median follow-up time for all patients was 4 years (range 6 months to 5 years).

Immunohistochemistry

Bladder samples were obtained at the time of diagnosis prior to any local or systemic treatment. The tissue was fixed in 10 per cent neutral buffered formalin and processed to paraffin wax. Paraffin sections were cut at 3 μm, dewaxed in xylene, and rehydrated in a graded series of alcohols. To block endogenous peroxidase activity, the sections were treated with 0·5 per cent H_2O_2 in methanol for 10 min.

The cripto-1 antibody used in this study was an affinity-purified rabbit polyclonal raised to a synthetic peptide (sequence: CPPSFYGRNCEHDVRKE). Its characterization and performance in immunohistochemical staining have been described previously.[6,8,10] After two washes in TBS, pH 7·6 (Tris buffered saline), the sections were incubated with 20 per cent normal swine serum in TBS for 30 min, followed by incubation with the primary antibody (1:40 in 20 per cent normal swine serum in TBS) for 1 h. After two washes in TBS, the sections were incubated with biotinylated swine anti-rabbit antibody diluted 1:1000 in TBS for 30 min, followed by another two washes in TBS before a 30-min incubation with horseradish peroxidase-conjugated streptavidin–biotin complex. The reaction was visualized by treating the sections with 0·1 per cent diaminobenzidine solution containing 0·02 per cent hydrogen peroxide for 5 min. The sections were counterstained with Mayer's haematoxylin. For each case, the following negative controls were included: one section with no primary antibody applied; one section with the primary antibody replaced by non-immune rabbit serum diluted to the same protein concentration as the primary antibody; and one section treated with the diaminobenzidine solution only.

All slides were examined by two pathologists (MCR and PB), who graded the extent and intensity of cripto-1 staining in the area of papillary tumour and in any normal, adjacent bladder tissue. In addition, areas of invasive tumour were assigned a separate staining grade. Staining was quantified depending on the percentage of immunopositive cells within each section (1+ = less than 30 per cent; 2+ = 30–60 per cent; 3+ = more than 60 per cent) and on the intensity of staining (1+ = weak staining; 2+ = strong staining). These two scores were then multiplied to give a final score between 1+ and 6+. A comparable scoring system has been used by other authors.[8,10] The clinical details and outcomes of individual patients were reviewed retrospectively. Results were analysed using the Minitab for Windows program. The χ^2 test was used to determine the statistical significance of the results. P values less than 0·05 were considered to be statistically significant.

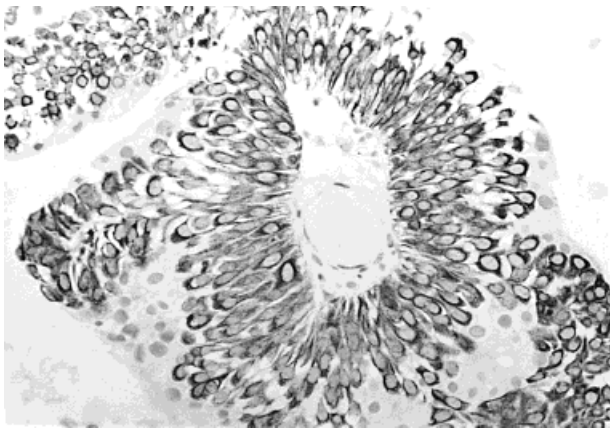

Fig. 1—Immunohistochemical detection of cytoplasmic cripto-1 in a section of human bladder cancer (magnification approximately × 400)

RESULTS

Bladder tumour sections

Cripto-1 staining was cytoplasmic and is demonstrated in Fig. 1. Thirty-nine patients (87 per cent) with bladder cancer had areas of papillary tumour on histological section. The remaining six cases of bladder cancer consisted only of invasive solid tumour. All 45 bladder tumours were positive for cripto-1 and the staining was homogeneous. Additional CIS, which was found in six of the 45 tumours, was positive for cripto-1, but the staining was more heterogeneous. Of the 21 specimens that contained adjacent histologically normal bladder, all demonstrated positive staining for cripto-1 (Fig. 2). Cripto-1 staining was compared between those with superficial (Ta/T1) and invasive (T2–T4) tumours, firstly in areas of normal benign bladder tissue (Table I, column A) and then in areas of papillary tumour where present (Table I, column B). There was no significant difference in the level of staining between the superficial and invasive tumours. There were no differences in staining between varying stages and grade combinations, such as TaG1 and T1G3 tumours. There was no correlation between tumour grade or stage and cripto-1 staining in either the superficial or the invasive groups.

Areas with 6+ cripto-1 staining were examined. Six of 21 (29 per cent) sections of histologically normal bladder adjacent to tumour had 6+ staining for cripto-1, compared with 23 of 39 (60 per cent) papillary bladder

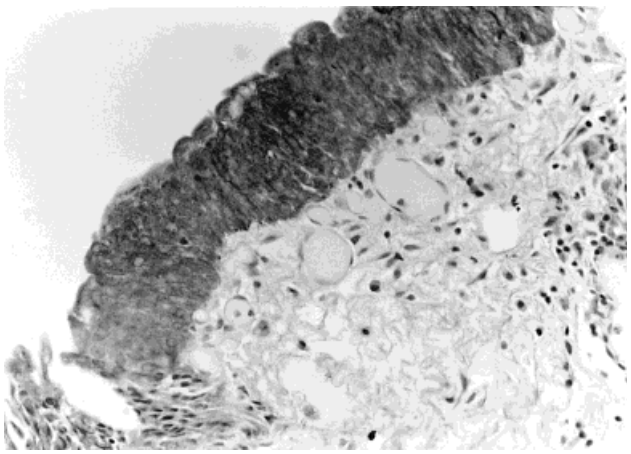

Fig. 2—Immunohistochemical detection of cripto-1 in histologically normal transitional epithelium adjacent to tumour (magnification approximately × 400)

Table I—Cripto-1 staining in malignant bladder specimens

Cripto-1 staining	(A) Normal bladder (No. of cases)		(B) Papillary bladder tumour (No. of cases)	
	Ta/T1	T2–T4	Ta/T1	T2–T4
1	0	0	2	0
2	6	5	4	3
3	1	1	3	4
4	1	1	0	0
6	6	0	17	6

Number of cases and levels of staining in histologically normal tissue (A) and papillary bladder tumour (B) are shown for superficial (Ta and T1) and muscle invasive (T3 and T4) cancers.

tumours ($P = 0{\cdot}025$). Patients who had 6+ staining in normal bladder adjacent to tumour showed no evidence of disease progression at the mean follow-up time of 5 years. Of the 23 patients who had 6+ staining for cripto-1 within the papillary tumour, three died of bladder cancer, three died of other causes, and nine had recurrent bladder tumour. The remaining eight patients had no recurrence. Of those who had less than 6+ staining in the papillary tumour, two died of bladder cancer, three died from other causes, and six had recurrent tumour. The remaining five patients had no recurrence. In the patients with muscle invasive disease, areas of invasive tumour within bladder sections were examined for the presence of cripto-1 staining (Table II). All areas of muscle invasion stained for cripto-1. There was no association between the level of cripto-1 staining in invasive areas of tumour and the clinical outcome or tumour grade.

Benign controls

None of the six benign controls demonstrated staining for cripto-1 (Fig. 3).

Table II—Cripto-1 staining in areas of muscle invasion in T2–T4 bladder tumours and associated clinical outcome

Cripto-1 staining	No. of patients	Clinical outcome			
		Dead CaB	Dead other	Controlled	Unknown outcome
1	2	1	0	1	0
2	3	1	1	0	1
3	8	3	3	1	1
4	2	1	0	1	0
6	4	2	0	1	1

Dead CaB = died from bladder cancer; Dead other = death from causes unrelated to bladder cancer; Controlled = control of disease at follow-up.

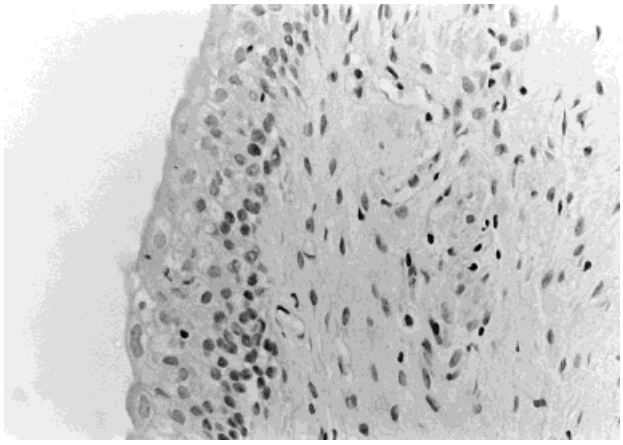

Fig. 3—Benign transitional epithelium from the control group with negative cripto-1 staining (magnification approximately × 400)

DISCUSSION

Histological grade and stage of bladder tumours at diagnosis are important prognostic parameters.[13,14] These parameters, however, will not always predict which patients with superficial (Ta/T1) tumours will progress to muscle invasive disease, or which patients with invasive disease (T2–T4) at diagnosis will develop distant metastases. The need to identify those patients at high risk of disease progression has led to the search for other prognostic markers within bladder tumours, one possible candidate being cripto-1.

Cripto-1 mRNA has been shown to be expressed in 64 per cent of primary or metastatic human colorectal tumours, but in only 2·6 per cent of normal colonic mucosa.[7] In human breast cancer cells, 75 per cent expressed the cripto-1 protein, while the adjacent non-involved breast epithelium was negative.[15] Qi *et al.*[8] detected cripto-1 by immunocytochemistry in only 13 per cent of normal breast tissue adjacent to breast tumour. Such findings suggested that cripto-1 may be a potential marker for breast cancer. A further study demonstrated cripto-1 immunoreactivity in most ductal cells, but its absence from most acinar cells in the normal human pancreas.[11] In human pancreas with chronic pancreatitis, although normal duct epithelial cells and

acinar cells did not stain with anti-cripto-1 antibody, cells arranged in duct-like glands within areas of pancreatitis were positive.[16]

In this study, a significant number of papillary bladder tumours (60 per cent) demonstrated intense (6+) staining for cripto-1 compared with areas of adjacent histologically normal bladder tissue (29 per cent). In addition, none of the benign bladder sections expressed cripto-1. This suggests that cripto-1 may have a role in the development and progression of bladder cancer. However, there was no association between clinical outcome and cripto-1 staining in areas of histologically benign bladder tissue, primary tumour, or tumour invasion, within malignant bladder sections. One could speculate that patients with bladder tumours and cripto-1 expression in adjacent normal tissue might have a worse prognosis. This would be due to alterations in cellular phenotype, possibly at the molecular level, manifested by altered cripto-1 expression but with no detectable histological change from normal urothelium. All patients with intense (6+) cripto-1 staining in adjacent normal bladder were alive and well at the 5-year follow-up, suggesting that cripto-1 expression in histologically normal bladder associated with tumour is not necessarily a poor prognostic factor. Similar studies staining for cripto-1 in breast tumours have also failed to demonstrate any significant correlation between the percentage of carcinoma cells that were positive for cripto-1 and oestrogen receptor status, axillary lymph node involvement, histological grade, tumour size, proliferative index, loss of heterozygosity on chromosome 17p, or overall patient survival.[8]

Bladder specimens from patients with inflammatory bladder disease, BPH, and normal bladders did not express cripto-1. This strongly suggests that the benign tissue adjacent to bladder tumours in this series, although histologically of normal appearance, is unlikely to be phenotypically normal. Although cripto-1 expression has been demonstrated previously in areas of pancreatic inflammation,[16] the two inflammatory bladder controls in this study were negative.

The exact role of cripto-1 in benign and malignant cells remains unknown. It has been suggested that it is associated with the de-differentiated state[4] and this is confirmed by the findings of the present study. Our results suggest a potential role for cripto-1 as a marker for bladder cancer and its possible association with the malignant phenotype prior to the histological diagnosis of carcinoma.

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