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An immunohistological demonstration of c-*erb*B-2 oncoprotein expression in primary urothelial bladder cancer

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Summary. Sections of formalin-fixed, paraffin-blocked tissue from 116 primary transitional cell carcinomas were stained immunohistochemically using a polyclonal antibody against the c-*erb*B-2 oncoprotein. Positive staining of cell membranes, known to correlate with gene amplification, was seen in 22 (19%) of the 116, with variable staining from tumour to tumour and within tumours themselves. Consistent with its mooted value as a prognosticator in bladder cancer, the c-*erb*B-2 oncoprotein was detected in 13 (of 40) grade III and 9 of the 26 muscle-invasive tumours examined compared to 1 (of 25) grade I and 6 (of 66) mucosa only (pTa) lesions. These results support further examination of c-*erb*B-2 expression in bladder cancer.

The c-*erb*B-2 gene, located on chromosome 17 at position q21 [15], is a normal cellular gene which encodes a protein with close sequence homology to epidermal growth factor receptor [1, 4] but with a distinct mitogenic intracellular signalling pathway [5]. This gene, first identified in its activated oncogenic form from rat neuroblastoma cells (hence the designation *neu*), is present in both normal and oncogenic forms. The oncogenic-*neu* gene has been shown to differ from the cellular-*neu* gene by a single base change (glutamic acid to valine) [1].

Overexpression of the c-*erb*B-2 gene oncoprotein has been demonstrated in a number of cancers by immunohistological as well as matrix blotting techniques, having been studied most extensively in breast cancer. Breast cancer patients, whose tumour cells stain positively for this tumour marker, have been suggested to have a worse prognosis both with respect to time to relapse and survival [3, 7, 11, 12, 14]. Consequently, c-*erb*B-2 expression has been proposed as a potential marker for inclusion in a predictive profile for breast cancer patients.

The aim of this study was to examine c-*erb*B-2 expression in transitional cell bladder cancer sections from our tumour bank to assess its potential as a useful prognostic marker in this disease.

Patients and methods

Primary transitional cell bladder tumours were obtained from 116 patients immediately following transurethral resection at Royal Brisbane and Toowoomba General Hospitals. Specimens were fixed in formalin then paraffin blocked prior to sectioning. The study group consisted of 95 males and 21 females with a mean age of 69 (± 13) years (range 30–94 years).

Control specimens were obtained as bladder biopsies from 13 patients who underwent cystourethroscopies for non-malignant conditions and whose bladders were cystoscopically and histologically normal. These patients' voided urines were normal bacteriologically and cytologically immediately prior to cystourethroscopy. This group consisted of 10 males and 3 females with an average age of 50 (± 17) years (range 16–70 years).

Preparation and staining

Paraffin sections (4 μm) were incubated in a warm oven at 37°C for 4–6 h. Following dewaxing and rehydration, the sections were washed in several changes of phosphate buffered saline (PBS) at pH 7.4. Non-specific antibody staining was inhibited prior to the commencement of staining by immersing the sections for 15 min in 3% non-fat skim milk powder in PBS. Excess blocker was removed by washing in PBS. The primary antibody (sheep anti-c-*erb*B-2 polyclonal; Cambridge Research Biochemicals, UK) was applied at 1:800 and the sections incubated overnight at room temperature in a humidified chamber. After this and subsequent incubations, the sections were again thoroughly washed in PBS. Biotinylated rabbit anti-sheep immunoglobulins [F(ab)2 fragment] 1:300 (Southern Biotechnology, USA) was applied for 2 h, then 1:250 streptavidin-horseradish peroxidase (Amersham, UK) for 45 min. Finally, the slides were developed in 3,3'-diaminobenzidine with H_2O_2 as substrate. The sections were counterstained with Harris' haematoxylin, dehydrated, cleared and mounted.

Negative control sections were incubated with PBS alone without primary antibody. Serial sections from those tumours which stained positively were stained as above, but the anti-c-*erb*B-2 antibody

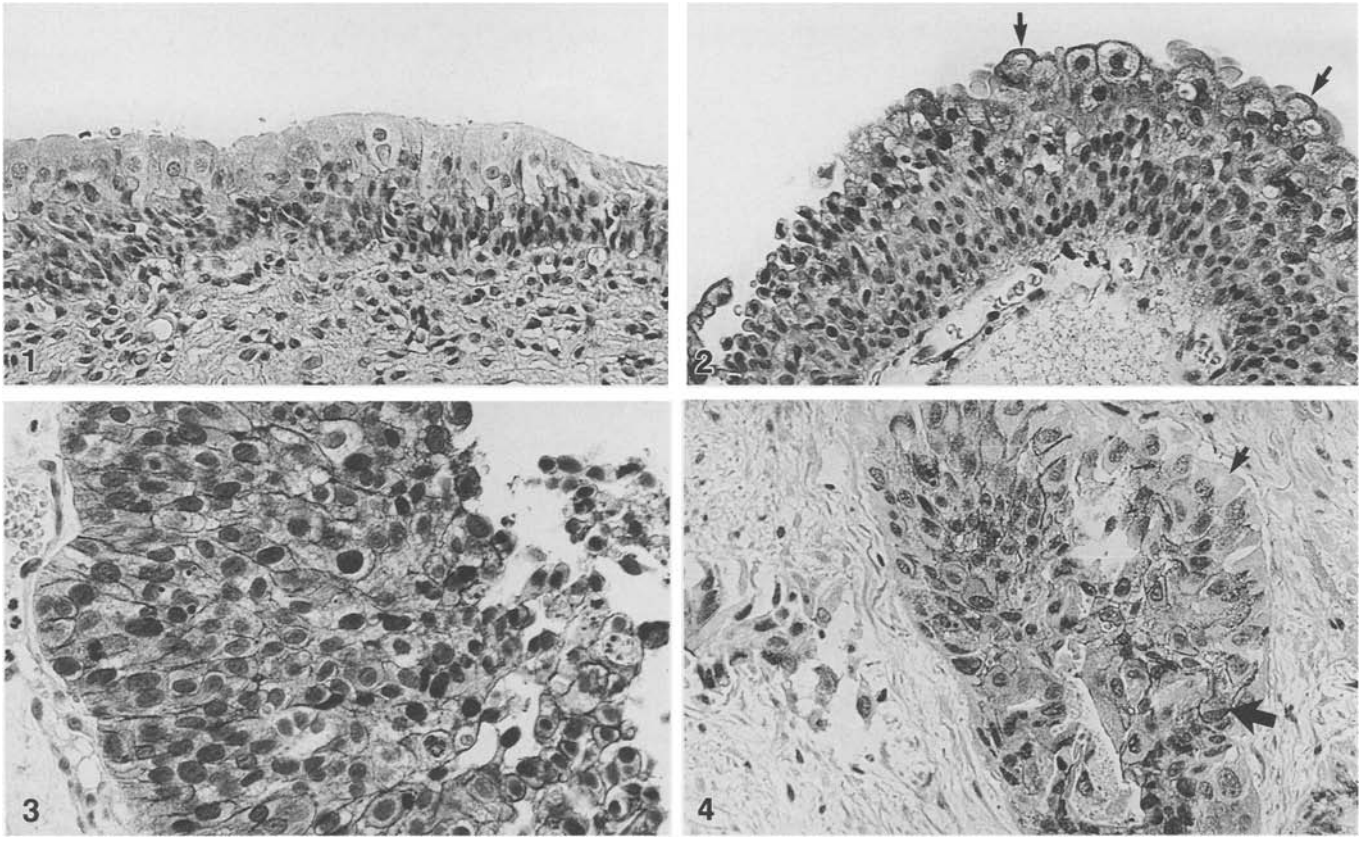

Fig. 1. Low-power photomicrograph of "normal" control bladder mucosa. ×200

Fig. 2. Low-power photomicrograph of grade II PTa transitional cell carcinoma with *arrowed* c-*erb*B-2 staining of umbrella cells. ×200

Fig. 3. Full-thickness staining for c-*erb*B-2 in a section of a grade II PTa bladder tumour ×200

Fig. 4. Patchy staining of an island of transitional cell carcinoma: *large arrow* pointing to cells with membrane staining and the *small arrow* to cells without staining ×200

was pre-absorbed with excess free immunogenic peptide for 4 h prior to application. Only those tumours in which the staining was abolished by this technique were regarded as expressing the gene product.

Assessment

Antibody-labelled tissue slides were examined as blinds, reference haematoxylin and eosin stained sections having been previously characterized for grade and pathological stage. A modification of the IUCC staging system was employed in this study, in which pTa signifies mucosa only lesions, pT1 those tumours involving lamina propria only, and pT2/pT2+ those in which invasion of muscle has been demonstrated.

Table 1. c-*erb*B-2 expression related to grade

Expression	Grade I	Grade II	Grade III
negative	24 (96.0%)	43 (84.3%)	27 (67.5%)
positive	1 (4.0%)	8 (15.7%)	13 (32.5%)
Total	25	51	40

Chi-square = 8.768; $P = 0.0125$

Table 2. c-*erb*B-2 expression related to pathological stage

Expression	Stage pTa	Stage pT1	Stage pT2/T2+
Negative	60 (90.9%)	17 (70.8%)	17 (65.4%)
Positive	6 (9.1%)	7 (29.2%)	9 (34.6%)
Total	66	24	26

Chi-square = 9.956; $P = 0.0069$

Results

c-*erb*B-2 expression was detected in 22 out of 116 (19%) primary bladder tumours, the details of which are outlined in Tables 1 and 2. None of the 13 "normal" control urothelial biopsies had detectable c-*erb*B-2 oncoprotein on their cells. Those tumours which displayed intense

staining of tumour cell membranes were regarded as expressing the oncogene protein, the significance of cytoplasmic staining being uncertain. There was considerable heterogeneity of expression between tumours and within individual tumours themselves, ranging from less than 10% of malignant cells staining positively to approximately 80%. The lower grade/stage tumours tended to have fewer positive cells with a more restricted distribution. The one grade I tumour found to express c-*erb*B-2 protein was positive only in the peripheral layer of cells. This pattern was also observed in 3 of the non-invasive grade II tumours. Only one of the superficial tumours exhibited full-thickness epithelial staining.

Considerable varation in distribution and intensity of staining was noted in the more aggressive tumours. In some cases only small groups of cells stained compared to other tumours in which nearly all the tumour mass was positive, but to differing degrees. In one grade III tumour which had squamous differentiation, the staining was restricted to the "islands" of squamous cells.

Discussion

Although a number of oncogene markers has now been evaluated in bladder cancer, none by itself has proved to be discriminating in terms of predicting natural history sufficient to warrant routine clinical use [6, 9, 10]. These oncogene markers, as "core indicators" of instigating gene-directed cell perturbations, promise to foretell imminent cellular dysfunction in relation to malignant behaviour.

Immunohistology is an attractive method for evaluating these potential oncogenic prognosticators, since this technique affords rapid assessment with identification of the cell types involved. Despite its subjectivity, immunohistological interpretation has been shown to have good correlation with southern blotting for c-*erb*B-2 in breast cancer [12, 13] with gene amplification having been shown to be closely associated with the gene product in both frozen and paraffin-embedded material [2, 13]. Cellular membrane staining was interpreted as a positive result, being consistent with c-*erb*B-2 protein's role as a transmembrane molecule. Cytoplasmic staining was not regarded as a positive result, occurring in some non-tumour as well as tumour cells. This pattern has been proposed to represent receptor ligand internalization [2, 8, 14].

In this study, only a relatively small percentage of tumours (22/116:19%) exhibited positive staining for c-*erb*B-2 in comparison to that reported for other oncogene markers in bladder cancer [6, 9, 10]. However, for c-*erb*B-2 expression, this finding is not very different to a reported incidence of approximately 30% in breast and ovarian cancers, considered to be those tumours in which c-*erb*B-2 oncoprotein expression is greatest [12, 14]. Since "sampling limitations" restricted the number of sections available from each tumour for immunohistology, the absolute number of tumours with some oncogenic c-*erb*B-2 expression may even be higher.

Consistent with the premise that expression of this oncoprotein is a marker of a more aggressive phenotype, 13 of the 22 (59%) positive tumour sections examined were grade III (with all but 3 of these muscle invasive) and 8 grade II (with half of these invading lamina propria) (Tables 1, 2). In addition, heterogeneity of staining for c-*erb*B-2 oncoprotein was present in this study, in terms of variability from cell to cell, area to area in the same section, and tumour to tumour. Such heterogeneity reflects the polyclonal nature of bladder cancer, which cannot be appreciated fully when routine histopathological examination alone is performed. However, in order to indicate accurately the number of cells exhibiting this marker in any one tumour, quantification is essential.

Identification of the contribution of a particular oncogene to a tumour has other ramifications apart from prediction of natural history. Since ultimately cancer is a disorder of gene transmission, documentation of molecular biological transgressions in malignant cells is an essential prerequisite before specific chemotherapy directed at gene level can be entertained. Overexpression of c-*erb*B-2 is thought to be an early event in tumorigenesis – albeit postinitiation – so identification of this tumour growth factor may be most pertinent in this regard too.

The results of this study support the contention that the c-*erb*B-2 oncogene product, together with other predictive parameters, may serve to provide a phenotypic profile which permits more accurate forecasting of bladder cancer behaviour, and may prove useful in the future as an important guide for directing specific anti-tumour therapy. Consequently, at this stage, apparent inapplicability of such markers in terms of current urological practice should not discourage continued documentation of their presence in bladder cancer.

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