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THE EXPRESSION OF C-ERB-B2 IN HUMAN UROTHELIAL CARCINOMA

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ABSTRACT

The expression of c-erb-B2 in human urothelial cancer was studied using an immunohistological (ABC) method. Staining characteristics of 86 tumors studied were analyzed with regards to grade, stage and outcome following treatment. Twenty-two, 9 and 20 tumors respectively were positive for c-erb-B2 out of 45, 15 and 26 G1, G2 and G3 tumors. Twenty-nine out of 55 pTa and pT1 tumors and 22 out of 31 muscle invasive tumors were positive for c-erb-B2. C-erb-B2 negative pTa and pT1 tumors were more commonly associated with no recurrence. Recurrences of higher grade and higher stage were more commonly associated with c-erb-B2 positive pTa and pT1 tumors. Survival appeared to be more common in the c-erb-B2 negative muscle invasive tumors in comparison with the c-erb-B2 positive tumors.

INTRODUCTION

Currently there is no accurate way to predict which superficial urothelial cancers will subsequently become muscle invasive or which muscle invasive urothelial tumors will subsequently progress and result in death. Studies regarding the immunohistological expression of c-erb-B2 by urothelial cancers are few and these studies have suggested varying rates of expression. The c-erb-B2 gene (also known as neu or HER2) located on chromosome 17q21 encodes a membrane bound glycoprotein (185 kDa transmembrane phosphoglycoprotein) which has sequence similarity with the epidermal growth factor receptor (c-erb-B1)[1]. The incidence of gene amplification seems to be greater for ERBB2 then epidermal growth factor (EGFr). Reported candidate ligands are the neu differentiation factor (NDF or Heregulin-α [HRGα]). Gorgoulis and co-workers[2] demonstrated amplification of ERBB2 in about 11% (4/35) of tumors. Amplification and over expression of c-erb-B2 (ERBB2) in carcinoma of breast and ovary is reported to be associated with inferior prognosis[3,4]. It has been suggested that in bladder cancer a weak association exists between staining for c-erb-B2 and tumor stage but no correlation was found earlier between c-erb-B2 and histological grade[5]. A weak positive association between p53 positivity and positive staining for c-erb-B2 has been suggested to exist in bladder cancer[5].

This study was initiated to test the hypothesis that expression of c-erb-B2 in urothelial cancer is more commonly associated with tumors of high grade and high category and that the expression of c-erb-B2 is associated with inferior outcome.

PATIENTS AND METHODS

Between 1990 and 1994, 86 patients (49 male and 37 female) with urothelial carcinomata, mean age 69.5 years (range 20 to 95

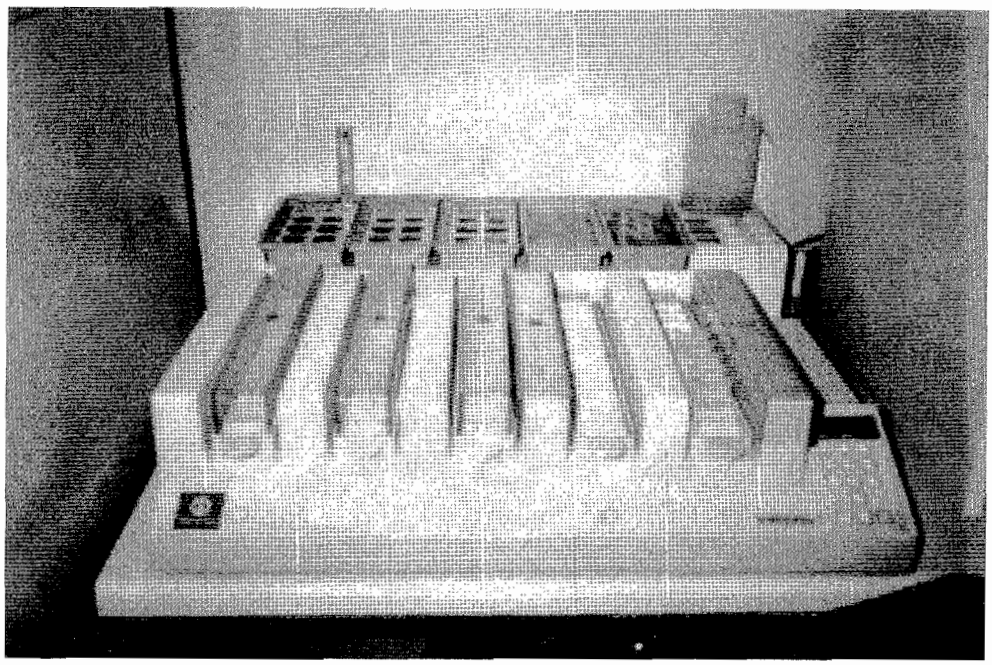

Fig. 1: Shandons Sequenza Immunostaining Centre

years) who were treated at Dryburn Hospital were enrolled in the study. These patients had a mean follow up time of 55.7 months.

Urothelial tumor samples were obtained from all the 86 patients requiring surgical excision or transurethral resection of their tumors. Fifty-five of these patients required transurethral resection of bladder tumors and 31 patients had resection of bladder tumors followed by radiotherapy and/or laparotomy and cystectomy. In each case, the tumors were staged based upon the TNM classification (UICC, 1987) by a careful bimanual examination under anaesthesia at the time of surgery in combination with the histology report. The tumors were graded according to the system of Bergkvist et al., 1965[1], using routine haematoxylin and eosin (H&E) stained sections of formalin fixed-paraffin embedded tumor.

The patients were followed up at regular intervals and any recurrent or persistent tumor carefully graded and staged (categorized). In the case of pTa and pT1 tumors these patients had 3 monthly check cystoscopies inititally for 2 years and in the absence of recurrence, check cystoscopies were carried out at 6 monthly intervals for 2 years following, while the patients were followed up at yearly intervals in the case of no recurrence. When a recurrent tumor was found, the follow up interval was reduced to 3 monthly intervals. Intravenous urography was performed at 2 yearly intervals and any recurrent or persistent tumor carefully graded and staged. The patients who had cystectomy were followed up in the outpatients department (these patients had careful clinical examinations and appropriate investigations as was indicated for example; bone scan, chest X-ray, liver function tests, intravenous urography, biopsy of any recurrent tumor as well as other investigations and management that were necessary). Those patients who had transurethral resection of their tumors and subsequent radiotherapy were followed up by regular check cystoscopies and bimanual examination. In the case of the patients with superficial bladder tumors who had frequent superficial recurrences, these patients were treated by intravesical chemotherapy following transurethral resection of their tumors.

Routinely, formalin fixed paraffin wax embedded blocks of urothelial cancer were cut at 5u and attached to poly-1-lysine coated

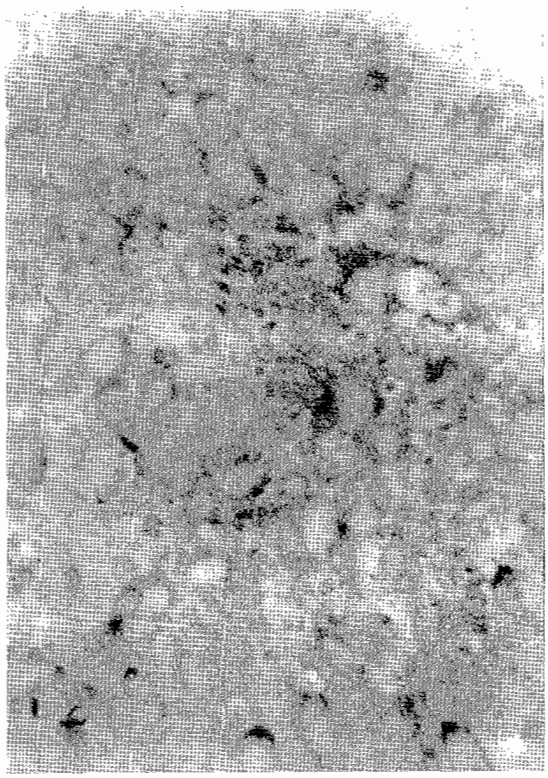

Fig. 2: Micrograph showing bladder carcinoma moderately positively stained for c-erb-B2

slides. The sections were allowed to dry overnight at room temperature. The following Avidin-Biotin peroxidase (ABC) immunocytological procedure was then carried out: The sections were deparaffinized, rehydrated, rinsed in tap water for 5 minutes and then rinsed in distilled water. Endogenous peroxidase activity was blocked by placing the sections in 1% hydrogen peroxide/methanol solution for 20 minutes. The sections were next rinsed in running tap water for 5 minutes. In order to allow for 'batch' runs to be performed and to provide consistent reproducible results, Shandons sequenza immunostaining centre and its cover plate assembly (Fig. 1) was used for the subsequent intermediate steps of the immunohistochemistry. In fact, the slides were put in the Shandons sequenza immunostaining centre and then rinsed in phosphate buffered saline (PBS) pH 7.2. Incubation was then carried out in normal rabbit serum (DAKO X902) diluted 1/20 with PBS for 10 minutes. The slides were next transferred to the primary antisera (Novo Castria NCL-CB11 anti-c-erb-B2 oncoprotein mouse monoclonal antibody) diluted 1/40 and incubated overnight at 4°C. The slides were next rinsed in PBS for 5 minutes and then incubated in secondary antisera (DAKO E354 rabbit anti-mouse immunoglobins/Biotinylated) diluted with PBS for 5 minutes and then incubated in AB complex (DAKO K 355 AB Complex/HRP) diluted 1/50 with PBS for 45 minutes and rinsed in PBS for 5 minutes. Immunoreactivity was visualized with Diaminobenzidine Tetrachloride dihydrate (DAB) solution for 1 to 5 minutes. The slides were rinsed in PBS for 5 minutes and then removed from the sequenza immunostaining centre and rinsed in running tap water for 10 minutes. The cell nuclei were counter stained lightly in Mayers Haematoxylin. The slides were washed, dehydrated, cleared and mounted in DPX.

Sections of formalin fixed paraffin wax embedded known c-erb-B2 positive breast cancer specimen were also cut at 5u and stained simultaneously with the urothelial tissues using the same steps as above for use as control slides. For purposes of negative control, sections of tumor specimens were processed and stained as above apart from the omission of the primary antisera.

Microscopy: (immunohistochemistry for c-erb-B2)

Routine microscopy for the immunohistochemistry slides was performed in order to assess each slide for the expression of c-erb-B2 (staining for c-erb-B2). Positive staining was demonstrated by peripheral (or membranous) staining but in addition, there was cystoplasmic staining in a few cases which were noted but not analyzed separately (Fig. 2).

Assessment of Staining for c-erb-B2

Staining was assessed taking into consideration the intensity of positive staining throughout the section. Staining intensity was scored on a 4 point scale: negative stain (no staining) = 0; weak = 1; moderate = 2 and strong = 3. The extent of staining was based upon the proportion of tumor cells positively stained: 0-25% (+), 25-50% (++), 50-75% (+++), 75-100% (++++). In the final analysis of data, all tumors showing moderate and strong

Table 1: The Expression of c-erb-B2 in Human Urothelial Carcinoma. Grade and Category.

	c-erb-B2 Positive	c-erb-B2 Negative	Totals
Grade			
Grade 1 (G1)	22	23	45
Grade 2 (G2)	9	6	15
Grade 3 (G3)	20	6	26
Totals	51	35	86
Category			
pTa and pT1 tumors	29	26	55
T2-T4 tumors	22	9	31
Totals	51	35	86

staining were recorded as positive and those showing no staining (0) or weak/mild staining were recorded as negative.

Statistical Analysis

Statistical analysis of the results was done using SPSS for Windows to calculate chi square tests of the various tumor groups and the outcome.

RESULTS

The results of the histological and immunohistochemical analysis of the tumors are summarized in Tables 1 and 2. Forty-five tumors were well differentiated (G1), 15 tumors were moderately differentiated (G2) and 26 tumors were poorly differentiated (G3). Thirty-one tumors were muscle invasive tumors and the remaining 55 tumors were pTa and pT1 tumors. Positive staining (medium and strong staining in $\geq$ 10% of tumors) for c-erb-B2 was obtained in 51 out of the 86 tumors studied and negative staining (no stain or mild staining) for c-erb-B2 was recorded in 35 tumors.

Grade and Category (Stage)

Twenty-two of the 45 G1 tumors were positive for c-erb-B2 and 9 of the 11 G2 tumors were positive for c-erb-B2. Twenty of the 26 G3 tumors were positive for c-erb-B2. The difference in staining characteristics of the well differentiated and the poorly differentiated tumors is significant (G1/G3 chi sq., $p<0.05$). Twenty-nine of the 55 pTa and pT1 tumors were positive for c-erb-B2 and 22 of the 31 T2 to T4 tumors were positive for c-erb-B2. The difference in the staining characteristics of the pTa and pT1 tumors in comparison with the T2 to T4 tumors is significant (Chi sq., $p<0.05$).

Outcome of pTa and pT1 as well as T2 to T4 (muscle-invasive) Tumors

Of the 20 pTa and pT1 tumors which did not recur, 16 were c-erb-B2 negative and only 4 were positive for c-erb-B2. Of the 35 tumors that recurred 25 were c-erb-B2 positive and only 10 were negative for c-erb-B2. Regarding the 21 tumors in which there were recurrences of the same grade and stage, 14 (67%) were positive for c-erb-B2 (medium or strong positive staining for c-erb-B2) and the remaining 7 were negative for c-erb-B2. There were 6 (75%) c-erb-B2 positive tumors out of the 8 in which the recurrences were of higher histological grade but the same stage. There were 5 c-erb-B2 positive tumors (91%) out of the 6 tumors in which the recurrences were of a

Table 2: The Expression of c-erb-B2 and Outcome of pTa and pT1, as well as of T2-T4 Tumors

	c-erb-B2 Positive	c-erb-B2 Negative	Totals
Outcome of pTa and pT1 tumors			
No recurrence	4	16	20
Recurrence of same grade and stage	14	7	21
Recurrence of higher grade and same stage	6	2	8
Recurrence of higher stage ± higher grade	5	1	6
Totals	29	26	55
Outcome of T2-T4 (muscle-invasive) tumors			
Alive	3	4	7
Died as a result of tumor	19	5	24
Totals	22	9	31

higher stage. Based upon the staining characteristics, the difference between the tumor groups in which there were no recurrences or the recurrences were of the same histological grade and stage on one hand and the tumor group with recurrences of higher grade and/or higher stage was significant ($p<0.001$).

Three patients out of the 22 with c-erb-B2 positive tumors were alive at the end of the study without any evidence of any recurrent or persistent tumor; the remaining 9 had died as a result of their tumors. In comparison, 4 patients out of 9 with c-erb-B2 negative tumors were alive at the end of the study. The remaining patients with c-erb-B2 negative tumors had died as a result of their tumors. The difference in the behaviour pattern of the two groups of tumors was significant (chi sq., $p<0.05$).

DISCUSSION

Studies concerning the expression of c-erb-B2 in urothelial carcinoma are few. In an initial study of 44 patients, positive staining of all intensities for c-erb-B2 protein product was observed in 36% of patients[6], although a previous study found little evidence of c-erb-B2 expression using paraffin embedded material and a different antibody[7]. In a study of 82 patients with primary transitional cell carcinoma of the bladder using the immunohistochemical method, strong or moderate staining for c-erb-B2 was found in 15% of tumors[5]. It has been stated that the weak correlation found between the expression of c-erb-B2 and tumor stage does not exclude a potential role as a prognostic factor, for in breast cancer also, no consistent correlation has been found despite a strong association between c-erb-B2 expression and poor survival[3]. In a series of 82 patients with transitional cell carcinoma, c-erb-B2 gene amplification assessed by Southern blotting was present in 14% and was associated with high grade (46% of grade 3 tumors showed amplification)[8]. In a study of 141 bladder tumor specimens, gene amplification for c-erb-B2, assessed using fluorescence in situ hybridisation (FISH), was detected in 7% of cases and was associated with c-erb-B2 over-expression[9]. However, over-expression without gene amplification was detected in 51 tumors. This and other studies[10] suggest that gene amplification is not a frequent cause of c-erb-B2 over-expression in bladder cancer. In carcinoma of the breast and ovary over-expression of c-erb-B2 was reported to be associated with gene amplification[11,12,14] and a poor clinical outcome.

In this study, urothelial tumors taken from 86 patients were examined by immunohistochemistry for c-erb-B2 using formalin fixed pa-

raffin embedded material and Novo Castria NCL CB11 mouse anti c-erb-B2 monoclonal antibody and Shandons Sequenza immunostaining center. Fifty-one out of the 86 urothelial carcinomas of all grades and categories (59%) examined were positive for c-erb-B2 (moderate or strong staining). An association between the expression of c-erb-B2 and grade and category was observed. (In the case of G1 and G3 tumors the difference in the expression rate of the tumors was statistically significant at $p<0.05$. In the case of pTa and pT1 tumors in comparison with T2 to T4 tumors the difference in the expression for c-erb-B2 was also statistically significant at $p<0.05$).

The observation that tumors associated with the expression of c-erb-B2 had worse outcome following radiotherapy and/or cystectomy in comparison with tumors negative for c-erb-B2 is important in view of the fact that the difference in the behaviour patterns was observed to be statistically significant. With this observation in mind, in the case of urothelial carcinoma, c-erb-B2 could be considered to be a marker of prognostication. Perhaps as an attempt to improve upon survival rates, patients who have c-erb-B2 positive T2 to T4 tumors should be treated by radiotherapy and adjuvant systemic chemotherapy or cystectomy and adjuvant systemic chemotherapy rather than radiotherapy alone or cystectomy alone.

Of importance is the fact that in the case of pTa and pT1 tumors most of the tumors that did not recur were c-erb-B2 negative and tumors that recurred were mostly c-erb-B2 positive. In addition, most of the tumors in which the recurrences were of higher grade or higher stage were c-erb-B2 positive. It could, therefore, be inferred from this further observation that pTa and pT1 tumors that are likely to progress have a high chance of being c-erb-B2 positive.

In previous studies, fresh frozen urothelial cancer specimens were used for immunohistochemistry for c-erb-B2, but in this study formalin fixed and paraffin embedded specimens have yielded good results.

Another important observation in this study is the fact that although the expression of c-erb-B2 had been associated with inferior prognosis, c-erb-B2 expression was not able to pick out completely all tumors that were associated with death of the patients. In view of this it may be a good idea to find out if another tumor marker could be used to identify c-erb-B2 negative tumors with the potential of being associated with grave outcome.

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