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Expression of c-*erb*B-2 Oncoprotein in Transitional Cell Bladder Cancer

P. Lipponen

Paraffin embedded tissue from 249 transitional cell bladder cancers (TCC) was stained by an antibody against c-*erb*B-2 oncoprotein to evaluate its overexpression. The staining results were related to histopathological features and clinical follow-up data. 99/249 (39%) of tumours were positive for c-*erb*B-2 oncoprotein and 31/249 (12.5%) of them showed moderate or heavy staining. c-*erb*B-2 overexpression was related to pelvic lymph node involvement ($P = 0.0355$) and distant metastasis ($P = 0.0058$) at the time of diagnosis, whereas no significant relationship was found between T-category and c-*erb*B-2 oncoprotein overexpression. Expression of c-*erb*B-2 was related to high WHO grade ($P = 0.0033$), DNA aneuploidy ($P = 0.0061$), high S-phase fraction ($P = 0.042$), and several morphometric nuclear factors ($P = 0.01$–0.09). All the tumours with high levels of c-*erb*B-2 expression were tetraploid in flow cytometry ($P < 0.0001$). c-*erb*B-2 expression predicted recurrence-free survival in superficial tumours ($P = 0.057$) and in survival analysis moderate or intense expression of c-*erb*B-2 oncoprotein was related to decreased survival probability ($P = 0.27$). In multivariate survival analysis overexpression of c-*erb*B-2 had no independent prognostic value. The results show that immunohistochemical demonstration of c-*erb*B-2 oncoprotein overexpression in paraffin embedded archival material has no prognostic value over already established predictors in TCC.
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INTRODUCTION

PROTO-ONCOGENES encode proteins that have a normal function, but when these genes are altered or expressed abnormally they are thought to contribute to the pathogenesis of cancer [1]. The proto-oncogene c-*erb*B-2 encodes a transmembrane growth factor receptor with significant sequence homology to the epidermal growth factor receptor, although they are not identical [2, 3]. Overexpression of the c-*erb*B-2/*neu* gene has been observed in breast [4, 5], ovarian [4] and lung [6] tumours and also in transitional cell bladder cancer (TCC) [7–11]. In breast [4, 5, 12, 13] and in ovarian [4] tumours amplification of c-*erb*B-2/*neu* is related to unfavourable prognosis, but the results are controversial. Alterations in oncogenes and oncoproteins have also been reported in bladder tumours [10–12] and there is a correlation between muscle invasive potential and epidermal growth factor receptors [15]. The importance of c-*erb*B-2 expression has not yet been fully evaluated in comparison to established prognostic factors in TCC and accordingly the aim of the present study was to investigate the expression of the c-*erb*B-2 oncoprotein in TCC. The results were correlated to previously characterised prognostic factors and clinical behaviour of TCC in a cohort of 249 patients followed-up for over 10 years.

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PATIENTS AND METHODS

Patients

249 patients with a newly diagnosed TCC were followed-up for a mean of 10.9 years (range 9.4–22 years) during 1965–1991. There were 199 males and 50 females and their mean age was 67 years at the time of diagnosis. The diagnosis, treatment and

follow-up of patients has been described previously [16, 17] and was conducted according to standard clinical practice [18]. The clinical staging of tumours was done according to UICC [19]. Progression was defined as an increase in T-, N-, and M-categories during the follow-up.

Histological methods

Transurethral or peroperative biopsy specimens from the primary tumours were fixed immediately after removal in buffered formalin (pH 7.0) and embedded in paraffin. For histological grading [20], 5 μm sections were cut and stained with haematoxylin and eosin. The growth type of tumours was identified and they were divided into papillary and nodular types. The distribution of patients into stage and grade categories is shown in Table 1. The methods of flow cytometry [17] and morphometry [16] have been presented in detail elsewhere.

c-erbB-2 immunohistochemistry

For immunohistochemical demonstration of c-*erb*B-2 oncoprotein, 5 μm sections from the primary TCC were deparaffinised and washed for 5 min with phosphate buffered saline (PBS) and treated with 0.5% pepsin in 0.1 mol/l HCl for 20 min at room temperature. The slides were covered with 3% normal horse serum in PBS for 15 min and then incubated overnight at 4°C with c-*erb*B-2 monoclonal antibody (Triton Biosciences Inc., U.S.A.) diluted 1:20 in PBS (91 sections were stained with this method). The remaining sections were stained using a monoclonal c-*erb*B-2 antibody (NCL-CB11) manufactured by Novocastra Laboratories (U.K.) and the dilution of this latter antibody was 1:100. No enzymatic pretreatment was done when the latter antibody was used since the staining results were similar whether enzymatic pretreatment was used or not. Sections were washed twice for 5 min with PBS, incubated for 20 min with horse anti-mouse biotinylated secondary antibody (Vector, California) diluted 1:200 in PBS. Slides were washed twice in PBS for 10 min and incubated for 20 min in pre-formed avidin–biotinylated peroxidase complex (ABC, Vectastain Elite kit, Vector, California). Sections were washed twice 5 min with PBS, devloped with diaminobenzidine tetrahydrochloride substrate (Sigman, U.K.), counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted.

Table 1. The cases subdivided according to c-erb B-2 oncoprotein expression T-category, WHO grade and DNA ploidy

		c-*erb*B-2 expression intensity				
Variable	Number	0	+	++	+++	*P* value*
T-category						
Ta-T1	119	77	29	8	5	
T2	69	35	23	7	4	0.8859
T3	38	24	11	2	1	
T4	23	14	5	3	1	
WHO grade						
1	97	68	25	2	2	
2	102	56	32	11	3	0.0033
3	50	26	11	7	6	
DNA ploidy						
Diploid	107	72	29	6	0	
Aneuploid	86	46	23	10	7	0.0064

*χ^2 test.

The staining of sections was evaluated by light microscopy with one observer at objective magnifications of 10 ×, 25 × and 40 ×. The intensity of staining was graded subjectively in a blinded manner into four categories: 0, +, ++, +++, as follows: 0, no positive staining; +, slight or weak staining (Fig. 1a); ++ moderate staining and +++ heavy or intense staining (Fig. 1b). These scoring criteria were determined as described previously [23] and the reader is referred to a colour plate in that reference. Staining was slight or weak when the cell membranes were clearly but weakly stained whereas staining was considered heavy when the cell membranes were visualised as dark and slightly thickened structures due to immunolabelling as shown in Fig. 1b. Staining was moderate when the cell membranes were clearly visualised, they were not thickened and the intensity of labelling was not as intense as in the heavy category. When the staining was evaluated, only membrane staining was considered positivity and cytoplasmic staining was not included in the scoring process. A negative control was included which never showed c-*erb*B-2 positivity. A positive control, which was a bladder cancer biopsy specimen showing intense (+++) positivity, was always positive for c-*erb*B-2 (Fig. 1b). The positive control showed similar staining whether or not enzymatic treatment was included and both antibodies gave similar staining results. The similarity of the staining result of the two antibodies

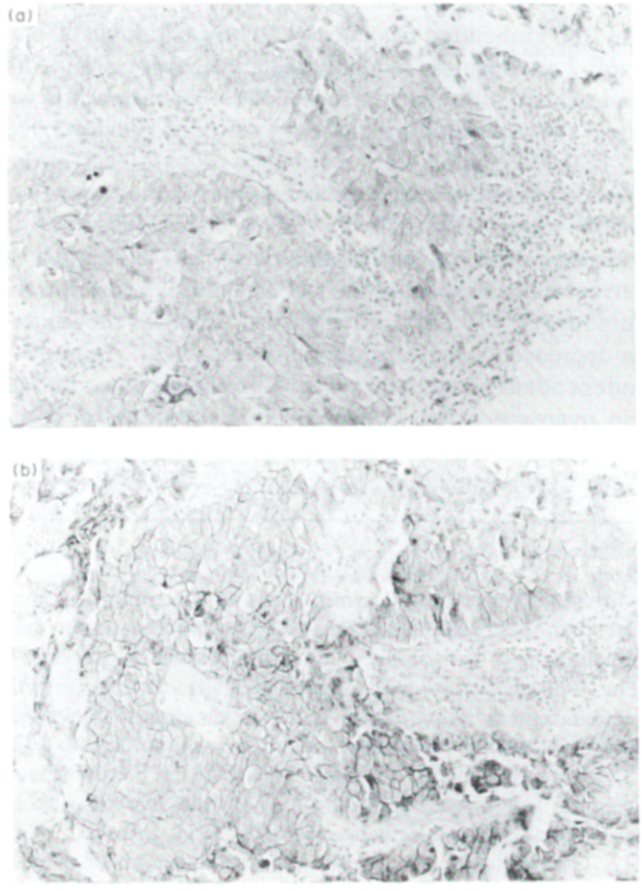

Fig. 1. (a) Immunohistochemical staining of c-*erb*B-2 oncoprotein in TCC. The expression of c-*erb*B-2 oncoprotein is weak at the cell membranes and the staining was scored weak (+) in this section. Magnification 400 ×. (b) Immunohistochemical staining of c-*erb*B-2 oncoprotein in TCC. The expression of c-*erb*B-2 oncoprotein is intense at the cell membranes and the staining was scored heavy (+++) in this section. Magnification 400 ×.

Table 2. The relationship between c-erb oncoprotein expression, pelvic lymph node involvement and distant metastasis at diagnosis

Variable	c-*erb*B-2 expression intensity: 0 and +	++ and +++	*P**
N0	195	23	
N1–2	23	8	0.0355
M0	212	26	
M1	6	5	0.0058

*Two-tail Fisher's exact test.

was assessed in 12 sections. The staining results of these 12 sections were as follows (Triton/Novocastra): negative (0) 4/4; slightly (+) positive 3/3; moderately (++) positive 3/3; intensively (+++) positive 2/2. The antibodies also showed similar cell membrane staining and the same regions in the consecutive sections were positive for c-*erb*B-2.

Statistical methods

The differences between the groups were tested using the SPPP/PC+ V3.0 programme package. Univariate survival analysis was based on life table (log-rank analysis) method with the statistics by Lee and Desu [21]. Multivariate survival analysis [22] was done with the BMDP (2L) programme package in a stepwise manner.

RESULTS

99/249 (39%) of the tumours were positive for c-*erb*B-2 oncoprotein and the majority of positive tumours (68/99, 68%) showed only weak immunolabeling. c-*erb*B-2 positivity (Fig. 1a, b) was usually focal and regionally variable in the sections.

c-*erb*B-2 positivity was not significantly related to papillary status ($P=0.2$) or to T-category (Table 1). The expression of c-*erb*B-2 oncoprotein was significantly related to pelvic lymph node metastasis and distant metastasis at diagnosis (Table 2). The significant relationship between WHO grade, DNA ploidy and c-*erb*B-2 overexpression is shown in Table 1. All the tumours ($n=7$) with heavy expression of c-*erb*B-2 oncoprotein were identified as tetraploid (DI 1.70–2.30) by flow cytometry ($\chi^2=23.8, P<0.0001$). The mean (S.D.) values of nuclear factors and proliferation indices categorised according to c-*erb*B-2 expression is shown in Table 3. Tetraploid tumours ($n=45$) with heavy (+++) c-*erb*B-2 expression showed identical S-phase fraction (SPF) and mitotic index values to remaining tetraploid tumours.

Table 3. The relationship between expression of c-erb B-2 oncoprotein, mean nuclear area (NA), S.D. of nuclear area (SDNA), nuclear perimetry (PE), longest nuclear axis (D_{max}), volume-corrected mitotic index (M/V index) and S phase fraction (SPF)

Variable	c-*erb*B-2 expression intensity: 0	+	++	+++	*P**
NA (SD), μm²	74 (26)	73 (22)	86 (40)	99 (23)	0.0134
SDNA (SD), μm²	23 (13)	21 (9)	28 (21)	37 (15)	0.0126
PE (SD), μm	33 (6)	34 (5)	36 (8)	39 (5)	0.0142
D_{max}, μm	12 (2)	12 (2)	12 (3)	14 (2)	0.0256
SPF, %	9 (9)	8 (8)	14 (11)	17 (8)	0.0196
M/V index	12 (14)	12 (12)	17 (15)	17 (12)	0.0999

*Kruskall–Wallis.

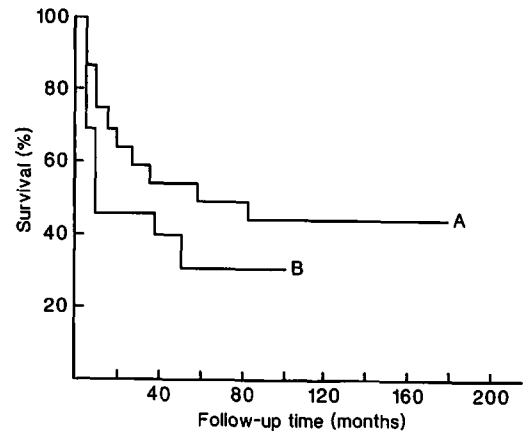

Fig. 2. The recurrence-free survival of Ta-T1 tumours categorised according to expression of c-*erb*B-2 oncoprotein. Curve A: absent (0) or weak (+), $n=104$; Curve B: moderate (++) or intense (+++) expression of c-*erb*B-2, $n=13$ ($\chi^2=3.7, P=0.057$).

Progression in T-, N-, or M- categories was not related significantly to c-*erb*B-2 overexpression ($P>0.2$). Progression in N- and M-categories in Ta-T1 tumours was related to c-*erb*B-2 expression, although only with a borderline significance. 14/105 (13%) of tumours with absent or weak (+) c-*erb*B-2 expression showed progression in M-category whereas 4/13 (31%) of tumours with moderate (++) or intense (+++) c-*erb*B-2 expression showed progression ($\chi^2=2.7, P=0.09$). Recurrence-free survival in superficial tumours was related to c-*erb*B-2 expression with a borderline significance (Fig. 2). In univariate survival analysis c-*erb*B-2 overexpression had only suggestive prognostic value (Fig. 3). The results of survival analyses in Ta-T1 tumours and in papillary tumours were also non-significant ($P>0.2$). In multivariate survival analysis (included T-category, papillary status, WHO grade, DNA ploidy and the variables

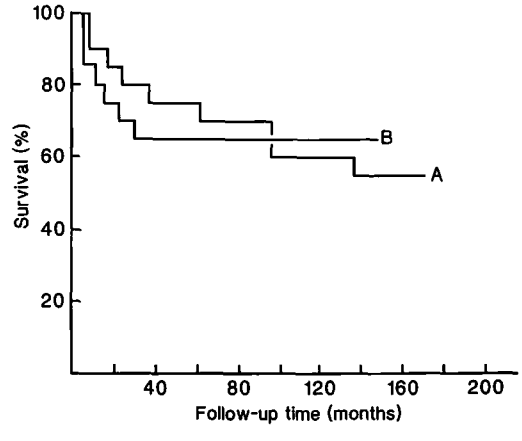

Fig. 3. The survival of patients categorised according to c-*erb*B-2 oncoprotein expression. Curve A: c-*erb*B-2 or 0 (+), $n=217$; Curve B: c-*erb*B-2 (++) or (+++), $n=31$ ($\chi^2=1.2, P=0.272$).

shown in Table 3) c-*erb*B-2 overexpression had no independent prognostic value over the standard prognostic factors [16, 17]. Independent predictors in the entire cohort were T-category [β(coefficient) =0.859, β/S.E. =6.251, $P<0.001$], papillary status (β=−0.788, β/S.E. =−2.684, $P=0.003$) and S phase fraction (β=0.028, β/S.E. =2.430, $P=0.023$).

DISCUSSION

The fraction of c-*erb*B-2 positive tumours is in line with the figures presented in previous reports [7, 9–11] and similar figures have been reported also in other epithelial tumours [4, 5, 12, 13]. However, in the majority of cases the expression of c-*erb*B-2 was weak and focal which may be related to biological factors as well as to methodological reasons. Previous analyses have shown that apparent expression is lower when paraffin embedded formalin fixed tissue is used and that a proportion of positive frozen sections may be negative when fixed material is used [7]. Recent analyses also show that overexpression of c-*erb*B-2 oncoprotein and gene amplification are not perfectly correlated [8, 11]. Accordingly, if the presence of the receptor at the cell membrane is responsible for biological effects, the evaluation of gene amplification alone may distort results when the relationship between c-*erb*B-2 gene and prognosis is evaluated. The dilution of antibody may also effect the number of positive tumours [13]. In this series several dilutions were tested and a dilution with a minimal background staining and a good cell membrane staining was used.

c-*erb*B-2 overexpression was significantly related to pelvic lymph node involvement and distant metastasis at diagnosis. These results are in agreement with previous results in TCC [10] and also in breast carcinomas c-*erb*B-2 overexpression is related to axillary lymph node involvement [13]. In this analysis no significant relationship was found between T-category and c-*erb*B-2 overexpression which is consistent with earlier reports [7, 9, 10]. Progression in T-category was independent of c-*erb*B-2 overexpression whereas progression in M-category was weakly related to moderate or heavy c-*erb*B-2 expression. This result further emphasises the relationship between c-*erb*B-2 expression and metastatic potential.

c-*erb*B-2 expression was clearly related to histological grading whereas no significant relationship was found between papillary status and c-*erb*B-2 overexpression. This suggests that the expression of c-*erb*B-2 is not always necessary for malignancy in TCC since nearly all nodular tumours are highly malignant [16, 17]. Expression of c-*erb*B-2 has also been found in normal cells [24] and in this analysis no significant difference was found in malignant features between c-*erb*B-2 negative and weakly (+) c-*erb*B-2 positive tumours. Survival analyses also suggest that only heavy expression has prognostic relevance [5, 12, 23]. The relationship between grade and c-*erb* overexpression has been described previously in TCC [9, 10, 23] as well as in other tumours [13].

DNA ploidy, proliferation indices, nuclear factors and c-*erb*B-2 overexpression were interrelated significantly. This is not unexpected since most of the malignant features are significantly interrelated in TCC [25]. The potential of c-*erb*B-2 overexpression to predict metastasis at diagnosis is probably based on the significant interrelationship between c-*erb*B-2 overexpression and cancer cell proliferation since proliferation indices owe a high potential to predict metastasis in TCC [17]. The relationship between nuclear size and c-*erb*B-2 overexpression was highly significant and in microscopic examination tumours with heavy positivity often showed large vesicular nuclei, which is line with the tetraploidy of these tumours [25].

These associations stimulate several questions such as, is the expression of c-*erb*B-2 a random phenomenon in TCC related to genetic regulatory mechanisms controlling nuclear structure and cancer cell proliferation or is it an independent phenomenon? The similar proliferation rates in c-*erb*B-2 positive and negative tetraploid tumours suggests that proliferation and expression of c-*erb*B-2 oncoprotein are independent features.

In survival analysis c-*erb*B-2 overexpression had no prognostic value over standard prognostic factors and the significance of morphometry, DNA flow cytometry and standard predictors has been discussed elsewhere [16, 17]. Immediately after diagnosis the survival curves were clearly separated (Fig. 3) due to the relationship between c-*erb*B-2 overexpression and metastasis at diagnosis. As the follow-up continued the curves crossed-over and no practical prognostic value was found for this oncoprotein. As far as the author is aware, long term follow-up studies with a large number of cases has not been reported previously. The prognostic significance has to be evaluated also in other cohorts and preferably by using frozen materials to avoid methodological confounding factors [7]. The prognostic significance of c-*erb*B-2 overexpression has been widely evaluated in breast cancer and the prognostic results are controversial particularly in local disease [4, 26, 27]. Most of the analyses have found that moderate or heavy expression of c-*erb*B-2 oncoprotein refers to lowered survival probability [12, 13] which concurs with the present results. Moderate or heavy expression of c-*erb*B-2 oncoprotein predicted recurrence-free survival in superficial tumours but the curves were not widely separated. This result has no practical meaning since progression in T-category could not be attributed to c-*erb*B-2 overexpression.

In summary, the results show that overexpression of c-*erb*B-2 oncoprotein in TCC is related to several malignant histological features and to metastatic behaviour at the initial phases of cancer development. No evidence was obtained that the expression of c-*erb*B-2 oncoprotein has significant prognostic value over standard prognostic factors in TCC.

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A Study of the Expression of Four Chemoresistance-related Genes in Human Primary and Metastatic Brain Tumours

Mireille Mousseau, Christiane Chauvin, Marie-France Nissou, Max Chaffanet, Dominique Plantaz, Basile Pasquier, René Schaerer and Alim Benabid

We investigated four mechanisms of intrinsic chemoresistance in a series of 67 human brain tumours including 31 gliomas (one grade I ganglioglioma, nine grade II and 10 grade III astrocytomas, 11 glioblastomas), 13 cerebral metastases, one medulloblastoma, one malignant teratoma, three ependymomas and 18 meningiomas. We studied four genes by northern blotting: multidrug-resistance (MDR 1), glutathione-s transferase (GSTπ), dihydrofolate reductase (DHFR), and topoisomerase II (Topo II). The Topo II gene was absent in the normal adult brain (100%) and in 64% of the tumour samples tested. A second gene, GSTπ, was found to be overexpressed in 38% of brain tumours. The two other chemoresistance-related genes were occasionally overexpressed in brain tumours (2% for MDR1, 9% for DHFR). Our results provide evidence that chemoresistance is intrinsic to the brain tissue and seems likely to be a multifactorial process.
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INTRODUCTION

BRAIN GLIOMA patients have an especially low 5 year survival rate, ranging from 0% to 38%, with a mean survival time of less than 1 year [1]. These tumours, because of their volume and/or their localisation, are often not accessible to locoregional treatments such as surgery or radiotherapy. Thus, numerous chemotherapy trials have been undertaken, with only low efficiency [1]. These disappointing results have been frequently attributed to the lack of efficient drug penetration across the blood–brain barrier [2]. This concept is discussed because the tumours disrupt the normal blood–brain barrier [2]. However, preclinical and clinical pharmacology data indicate that the drug concentration is often low in brain tumours but lower in the apparently normal brain tissue surrounding the tumour, which may contain infiltrating cancer cells [2]. These dispersed cells may represent the "stem cells" that can repopulate the tumour and that are the most chemoresistant cells [3]. We report here the results of our investigations on the mechanism of chemoresistance of brain tumour cells.

In this report, four main mechanisms of tumour cell resistance