**C-erbB-2 Gene Amplification: A Molecular Marker in Recurrent Bladder Tumors?**

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**ABSTRACT**

*C-erbB-2* gene amplification and protein overexpression have been implicated as prognostic markers for patients with recurrent progressive bladder tumors. This event has been investigated as a potential diagnostic indicator in archival samples of transitional cell carcinoma of the bladder. Two hundred thirty-six bladder tumors from 89 patients with recurrent disease (mean follow-up, 4 years), 20 tumors from patients with no evidence of bladder tumor recurrence (mean follow-up, 7 years) and 10 normal bladder controls (patients with no history of transitional cell carcinoma) were studied. A differential PCR was used to provide a semiquantitative estimate of *C-erbB-2* gene amplification. Protein overexpression was assessed immunohistochemically. Sixteen of 89 patients with recurrent disease had evidence of *C-erbB-2* gene amplification. No *C-erbB-2* gene amplification was seen in the nonrecurrent tumors or normal bladder controls. Of the 89 patients with recurrent bladder tumors, 43 had evidence of progressive disease, and of these, 14 patients exhibited *C-erbB-2* gene amplification, indicating a strong association with gene amplification and progressive disease (*P < 0.0005*). Gene amplification in these patients was seen only after disease progression had occurred. Protein overexpression was seen in 50% of patients with recurrent and 45% of patients with nonrecurrent disease. No protein overexpression was seen in normal controls. Protein overexpression could not be linked to disease progression. *C-erbB-2* gene amplification and protein overexpression were of predictive value in multivariate analysis for overall bladder cancer death; however stage and grade remained the most important independent prognostic variables. *C-erbB-2* gene amplification and protein overexpression were of no value as independent markers for the prediction of disease recurrence or progression. It appears from these results that the role of *C-erbB-2* as a diagnostic marker may far outweigh its usefulness as a prognostic indicator.

**INTRODUCTION**

Transitional cell carcinoma (TCC) is the fourth most common cancer affecting males within the United Kingdom, with a male:female ratio of 3:1. Between 1971 and 1984, the overall incidence of this disease rose by 31%, with a corresponding rise in annual mortality of 22%. From the data available, it appears that a subgroup of aggressive tumors are responsible for most morbidity and mortality (1–3). TCC is staged and graded according to the UICC criteria (1978). Superficial tumors have a good overall prognosis (90–100% 5-year survival), but once the tumor becomes muscle invasive, the prognosis worsens (20–40% 5-year survival; Ref. 4). Seventy % of patients with superficial tumors are at risk of recurrence, and approximately 10% of these patients progress to muscle-invasive disease. To date, there is no indicator to identify those patients at risk, and therefore, all patients undergo extensive follow up and multiple cystoscopies (5).

The *C-erbB-2* gene, located on chromosome 17q21, encodes a Mr 185,000 transmembrane phosphoglycoprotein. Structural studies of the *C-erbB-2* protein and the epidermal growth factor receptor show significant sequence homology and identical gross structural organisation in ligand binding, transmembrane, and tyrosine kinase domains (6–8).

To date, *C-erbB-2* gene amplification and protein overexpression are known to correlate strongly with node-positive breast carcinoma and overall reduction in disease-free survival (9, 10). Slamon et al. (11) have postulated that disease behavior differs for breast tumors with *C-erbB-2* gene copy numbers of 1, 2–5, 5–20, and greater than 20, indicating a possible need to assess accurately *C-erbB-2* gene copy number in human tumors.

Studies of *C-erbB-2* gene amplification and protein overexpression have shown varying results in bladder cancer. The proportion of cases reported to show *C-erbB-2* overexpression has ranged from 7 to 70%. *C-erbB-2* gene amplification appears to occur on a less frequent basis, with rates varying from 8 to 46%, indicating that other mechanisms may contribute to the elevated levels of protein expression. It has been shown that *C-erbB-2* gene amplification is found most frequently in muscle-invasive tumors, and it has been postulated that *C-erbB-2* gene amplification may be a true prognostic indicator for patients with progressive disease (12–15). The aim, therefore, of this study was to evaluate *C-erbB-2* as a marker for recurrence and disease progression in bladder tumors.

**MATERIALS AND METHODS**

**Patient Material**

Formalin-fixed, paraffin-embedded blocks of initial tumor and subsequent tumor recurrences were selected using Pathology department archives from patients over a 10-year period (1982–1992). Patients with a tumor that had not recurred over a 5-year period from initial diagnosis were classified as having nonrecurrent tumor. Bladder tissue from patients with no history of bladder carcinoma were used as normal controls. Full clinical details were available on all patients selected.

Two hundred thirty-six tumors from 89 patients with recurrent disease (mean follow up, 4 years), 20 patients with no evidence of disease recurrence (mean follow up, 7 years), and 10 normal bladder controls were analyzed. Tissue was processed routinely by the Department of Pathology. The majority of tissue was removed by electrocautery, or cold punch biopsy, fixed in 10% buffered formalin, and wax embedded. The bladder tumors were restaged and regraded by one pathologist using the UICC 1978 criteria so that the same pathological criteria were used throughout the study. One 10-μm section was cut for estimation of gene amplification, and two 5-μm sections were cut onto 3-amino-5-propyl triethoxysilane-coated slides for the immunohistochemical localization of the *C-erbB-2* protein. The percentage of tumor content was estimated, with all tissue analyzed having a tumor content of greater than 60%.

**Determination of Gene Amplification**

Gene amplification was assessed semiquantitatively using a differential PCR. This involves coamplification of a reference gene (single copy) and target gene (unknown copy) within the same reaction tube. The single copy reference:target gene relative ratio, when compared to cell line quality controls exhibiting varying copies of the *C-erbB-2* gene, gave a semiquantitative estimate of *C-erbB-2* gene amplification (16, 17).

**Cell Lines**

The SKBR3, MDA-MB-361, MDA-MB-453, and BT20 cell lines were acquired from the ATCC. The N87 cell line was acquired from the American Type Culture Collection.

Received 10/26/94; accepted 4/4/95.

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1. To whom requests for reprints should be addressed.

2. The abbreviations used are: TCC, transitional cell carcinoma; df, degrees of freedom.
PCR Conditions

Table 1). centriuged at 900 X g for 10 min. Cell pellets were resuspended in 1-2 drops ase, and 10X reaction buffer (Boehringer-Mannheim) were added. The reac centrifuged, and the tubes were placed on ice. Twenty-five ^1 of mastermix (2 then rapidly cooled to 4°C on a thermocycler (Biometra). Samples were pulse of archival DNA in a final volume of 25 fil and heated to 95°C for 10 min and Oligonucleotides
tion of the samples.

DNA Extraction

A 10-µm section from each archival tumor block was taken and placed in a sterile microfuge tube. The blade was changed between blocks, and the work surfaces continually cleaned with xylene to prevent cross-contamination. The
surfaces were handled using a cell scraper from a 175-cm2 flask into sterile PBS (10 mm sodium phosphate-140 mM sodium chloride, pH 7.6). The cell suspension was centrifuged at 900 × g for 10 min. Cell pellets were resuspended in 1-2 drops 1% agarose, fixed in 10% buffered formalin for 48 h, and then embedded in paraffin wax. Sections of this fixed material (10 µm) were processed to provide external standards for PCR analysis.

Cell Culture

All cells were cultured using the appropriate media, 10% heat inactivated FCS (MDA-MB-361, 15%), 100 units/ml penicillin, and 100 µg/ml streptomycin (GIBCO, Paisley, United Kingdom). Cells were grown in 5% CO2 in air at 37°C in a humidified incubator. Media used for culture were Leibovitz’s L-15 (MDA-MB-453 and MDA-MB-361), McCoy’s 5a medium (SKBR3), RPMI 1640 (N87), and Eagle’s MEM with nonessential amino acids and Earle’s balanced salt solution (BT20). Cells were grown to confluence and harvested using a cell scraper from a 175-cm2 flask into sterile PBS (10 mM sodium phosphate-140 mM sodium chloride, pH 7.6). The cell suspension was harvested using a cell scraper from a 175-cm2 flask into sterile PBS (10 mM sodium phosphate-140 mM sodium chloride, pH 7.6). The cell suspension was harvested using a cell scraper from a 175-cm2 flask into sterile PBS (10 mM sodium phosphate-140 mM sodium chloride, pH 7.6). The cell suspension was harvested using a cell scraper from a 175-cm2 flask into sterile PBS (10 mM sodium phosphate-140 mM sodium chloride, pH 7.6). The cell suspension was centrifuged at 900 × g for 10 min. Cell pellets were resuspended in 1-2 drops 1% agarose, fixed in 10% buffered formalin for 48 h, and then embedded in paraffin wax. Sections of this fixed material (10 µm) were processed to provide external standards for PCR analysis.

Oligonucleotides

Oligonucleotides were selected for the target gene C-erbB-2 generating a 98-bp fragment and for reference genes as defined by Neubauer et al. (Ref. 17; Table 1).

PCR Conditions

Competitive PCRs were carried out in a total volume of 50 µL. For individual amplitmer pairings, see below ("Analytical Algorithm"). Prior to the PCR, 0.5 µL of each oligonucleotide (four in total) were combined with 10 µL of archival DNA in a final volume of 25 µL and heated to 95°C for 10 min and then rapidly cooled to 4°C on a thermocycler (Biometra). Samples were pulse centrifuged, and the tubes were placed on ice. Twenty-five µL of mastermix (2 mM each of dATP, dTTP, and dGTP, 0.5 mM of dCTP, Pharmacia), 1 µCi [α-32P]dCTP or 1 µCi [α-33P]dCTP (Amersham), 1 unit Taq DNA polymerase, and 10X reaction buffer (Boehringer-Mannheim) were added. The reac- tion mixture was overlaid with two drops of mineral oil. PCR conditions were: cycles 1-24, 94°C for 1 min; 51°C for 1 min; 72°C for 1 min; and cycle 25, 94°C for 1 min; 51°C for 1 min; and 72°C for 10 min, followed by incubation at 60°C for 30 min.

The radiolabeled PCR products generated were resolved by electrophoresis on a 10% polyacrylamide gel. The gel was transferred onto Whatman’s 3MM paper and dried under vacuum for 50 min. The products were visualized by autoradiography with Min-R PE (Kodak) film at -70°C.

Analytical Algorithm

To analyze gene copy number for C-erbB-2, an analytical algorithm as described by Neubauer et al. (17) was used (Fig. 1). In this algorithm, a series of competitive PCRs are performed to confirm gene amplification and to exclude spurious results.

Reaction 1: IFN-γ 150-bp and IFN-γ 82-bp Competitive PCR. DNA extracted from archival material is known to be fragmented (20–22). This reaction was carried out to confirm that extracted DNA was of sufficient quality to allow further analysis. In any competitive PCR, preferential amplification of shorter PCR products occurs. Highly fragmented DNA would contain significantly more targets for the IFN-γ 82-bp primers than the IFN-γ 150-bp primers and could also give spurious results in subsequent competitive PCRs. The reaction generates two different-sized fragments, each from a different exon of the IFN-γ gene. Differences in signal intensity of the two bands could not be attributed to gene amplification but would result from DNA fragmentation. The GC content, and therefore the labeling index, differs significantly (40 versus 23%); IFN-γ 150-bp versus 82-bp for these products; after correction for this factor, DNA exhibiting a IFN-γ 82-bp:150-bp ratio of greater than 3:1 was excluded from further analysis. Neubauer et al. (17) found DNase digested genomic DNA, resulting in IFN-γ 150-bp versus 82-bp ratios of 4 still able to be analyzed for C-erbB-2 gene amplification using the C-erbB-2 98-bp:IFN-γ 85-bp ratio.

Reactions 2 & 3: IFN-γ 150-bp and C-erbB-2 98-bp Competitive PCR. These reactions provide the screen for gene amplification of the target C-erbB-2 using IFN-γ as a reference gene. The assumption was that IFN-γ was not amplified in any of the tissue samples. Reaction 2 screened C-erbB-2 amplification against the larger amplimer, IFN-γ 150-bp. Potentially, this reaction may produce false positive results, should the smaller C-erbB-2 PCR product undergo a more efficient process of amplification than the larger IFN-γ. Reaction 3 was performed to eliminate this possibility. The IFN-γ 85-bp amplimer amplifies the same exon of the IFN-γ gene, using the same 3’ primer as the IFN-γ 150-bp reaction. This reduces variation between these reactions that might otherwise complicate the analysis. The combination of these two reactions provides a stringent screening procedure for amplification of C-erbB-2 in relation to the reference gene IFN-γ.

Reaction 4: N-ras 110-bp versus C-erbB-2 98-bp Competitive PCR. Genetic instability is a function of many tumors. The potential for chromosomal deletion or replication involving the IFN-γ reference gene used above, therefore, exists. To exclude the possibility that deletion or replication of the IFN-γ gene might bias results in some aneuploid tumors, an additional reference gene from a different chromosome was used. Competitive PCR with N-ras 110-bp versus C-erbB-2 98-bp was performed on all amplified tumors and a proportion of nonamplified tumors. Only tumors that showed amplification in all three test reactions (Reactions 2, 3, and 4) were scored as amplified (Fig. 2).

Table 1. Sequences of the oligonucleotides used as PCR primers in this study

<table>
<thead>
<tr>
<th>Amplimer size</th>
<th>5’-primer</th>
<th>3’-primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-erbB-2 98-bp 2122-2219 (Ref. 23)</td>
<td>5’- CCTTCGAGCCTGAGCTGCTGCCT-3’</td>
<td>5’- ATCCTTTCGCTGAGCTGCTGCCT-3’</td>
</tr>
<tr>
<td>IFN-γ 150-bp 4502-4731 (Ref. 24)</td>
<td>5’- TCTTTCCTTTTCCTCCGATAGGT-3’</td>
<td>5’- GTGGAGGCTCTCGCTGCGCTC-3’</td>
</tr>
<tr>
<td>IFN-γ 85-bp 4647-4731 (Ref. 24)</td>
<td>5’- AGTAGGCTCCGATGCTGCTGC-3’</td>
<td>5’- TCGGAGGCTCTCGCTGCGCTC-3’</td>
</tr>
<tr>
<td>IFN-γ 82-bp 2012-2093 (Ref. 24)</td>
<td>5’- GCAAGGCCAATATGGCTTCTCTC-3’</td>
<td>5’- GTCCTCCACCATCTCTTGGCA-3’</td>
</tr>
<tr>
<td>N-ras 110-bp 1-111 (Ref. 25)</td>
<td>5’- AGACTGCTGGTACAACTGGTT-3’</td>
<td>5’- CTCCTAGTGCGGATCATATT-3’</td>
</tr>
</tbody>
</table>
Estimation of Copy Number cell lines were selected with increasing copy numbers of the C-erbB-2 gene. To allow a degree of quantitative analysis to be performed, to this end, it was possible to produce a quantitative estimate for the degree of C-erbB-2 gene amplification in each individual tumor sample. These external standards were also used to assess the reproducibility, sensitivity, and accuracy of this method in multiple experiments. The experiments used to characterize the cell lines were performed over a 3-day period using both [32P]dCTP and [12P]dCTP. Data from these experiments enabled us to semiquantitatively estimate C-erbB-2 gene copy number of tumor samples when using the cell lines as external quality controls (26).

**C-erbB-2 98-bp:IFN-γ 85-bp versus Copy Number.** The C-erbB-2 98-bp:IFN-γ 85-bp ratio reaction product to gene copy number was established using the cell lines described. (See “Materials and Methods”). A linear relationship between these parameters was observed when multiple samples from each cell line were analyzed (Fig. 4).

**Sensitivity.** To determine the sensitivity of the assay system in detecting differences in copy number, samples of the cell lines BT-20 (1 copy, C-erbB-2), MDA-MB-453 (2 copies), MDA-MB-361 (2–4 copies), SKBR3 (4–8 copies), and N87 (>8 copies) were included in the assays using both [32P] and [12P]; they were performed over a 3-day period. In all cases, the mean ±2 SDs were estimated to provide the 95% confidence intervals for each of the cell lines (and therefore the relative copy number for C-erbB-2). These results are shown in Table 2.

Samples whose ratios for PCR Reaction 3 fell within the range of one of the above cell lines were assigned a copy number identical to the cell line; those falling between the ranges of two cell lines were assigned a copy number between the copy numbers of the two cell lines. For example, in assay 1 above, a sample with a ratio of 1.24 would fall within the range found for MDA-MB-453 and would be assigned a copy number of 2, while a sample with a ratio of 1.6 would fall between the ratios of MDA-MB-453 and SKBR3 and be assigned a copy number of 2–4. After analysis of intra-assay and inter-assay data, the cell lines, when used as external quality controls, were run in triplicate with each set of tumor samples (26).

**Immunohistochemistry**

To identify expression of the C-erbB-2 oncoprotein, the antibody used was an affinity-purified rabbit polyclonal raised to a synthetic peptide of the human C-erbB-2 oncoprotein (Dako, High Wycombe, United Kingdom). Sections were cut, dewaxed, and rehydrated; tissue endogenous peroxidase was blocked by treating the sections with 3% hydrogen peroxide in 10 mM sodium phosphate-140 mM sodium chloride, pH 7.6 (PBS). A routine streptavidin-biotin

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**Fig. 1.** Analytical algorithm used to analyze gene copy number for C-erbB-2.

**Fig. 2.** Autoradiograph demonstrating C-erbB-2 gene amplification. Patient J demonstrates gene amplification comparable to SKBR3, Patient K demonstrates gene amplification comparable to MDA-MB-453, and patient K demonstrates no C-erbB-2 gene amplification.

**Fig. 3.** Autoradiograph demonstrating C-erbB-2 gene amplification by differential PCR for all cell lines. BT20, 1 copy; MDA-MB-453, 2 copies; MDA-MB-361, 2–4 copies; SKBR3, 4–8 copies; N87, >8 copies.
system was used to detect protein overexpression. To reduce nonspecific binding, sections were incubated with blocking solution containing 25% human and 25% swine serum in PBS. After 15 min, the blocking serum was aspirated, and the sections were incubated with the primary antibody at a dilution of 1:200 made up in 10% human serum and 10% swine serum for 60 min at room temperature. After three 5-min washes with PBS, the sections were incubated with the secondary antibody, a biotinylated swine anti-rabbit immunoglobulin at 1:200 in 10% human and 10% swine serum (Dako) for 40 min at room temperature. After three 5-min washes in PBS, a horseradish peroxidase-conjugated streptavidin-biotin complex was applied. Peroxidase activity was demonstrated using freshly prepared 0.05% diaminobenzidine tetrachloride with 0.01% hydrogen peroxide. Nuclei were counterstained with hematoxylin, and the slides were dehydrated and mounted. Isotopic controls were processed with every tumor using nonimmune rabbit immunoglobulins in the place of the primary antibody. A formalin-fixed breast carcinoma was used as a positive control and included with every batch processed. Sections were classified by a second observer, and in cases of discrepancy, revised. Only tumors displaying clear membrane staining were classified as positive, representing C-erbB-2 protein overexpression. The intensity of staining was further subclassified after comparison with the positive control as 1+ (weak membrane staining) and 3+ (strong membrane staining).

Clinical Course

Forty-seven of the patients with recurrent disease died during the study period (53%). Thirty-three of those died of bladder cancer. There were no deaths in the nonrecurrent group. Median age of patients was 72 years (52–94 years), with a male:female ratio of 2.5:1. Median follow up was 71 months (range, 3–300). To try to overcome the problem of taking biopsy material from residual tumor at the first 6-week follow-up cystoscopy, tumors were classified as recurrences only if the first follow-up cystoscopy was clear. We were unable to use size and multiplicity of tumors as prognostic variables because operative notes and clinical follow up in many cases for these factors were poorly documented in the clinical records. Disease progression was defined as a worsening stage histologically and/or clinical evidence of metastatic disease. Twenty-one patients with superficial tumors at presentation progressed, and 22 patients presented at initial diagnosis with muscle-invasive disease. This group of patients was categorized as having disease progression. In all, this accounted for 43 patients with evidence of disease progression.

Statistical Analysis

The associations between gene amplification and protein overexpression with clinical and histological variables were assessed by means of $\chi^2$ and Fisher's exact tests. The influence of potential prognostic indicators on event-free survival was estimated by Cox proportional hazards model (27). Variables examined included age, sex, stage, grade at presentation, protein overexpression at presentation, and gene amplification. Each factor was first tested as a single regression variable in the model (univariate). A stepwise multivariate regression approach with backward elimination was then used to identify the most important predictor variable with respect to event-free survival. $P < 0.05$ after adjustment for the effects of other variables was required for retention in the model. For the Cox regression analysis, $\exp(\beta)$ gave a point estimate for the relative hazard. For age, the relative hazards are presented as the hazard increase per 10-year advance in age. The relative hazard for sex was given as male versus female and stage/grade coded for worsening stage and increasing grade. Kaplan-Meier survival curves split by gene amplification and protein overexpression were plotted and analyzed using the log rank method (28). In survival models, the data from patients who died of causes other than bladder cancer and who were free of tumor at that time were treated as censored data. This data was analyzed by use of the CSS Statistica Stats Soft, Inc. statistical software package.

Pathology

A total of 236 tumors from 89 patients with recurrent disease were suitable for analysis. Mean tumors per patient was 2.65 (range, 1–10). The documented stage and grade relates to the primary tumor from each patient. One hundred sixty-nine tumors were confined to the bladder mucosa (pT1), 36 showed invasion of the lamina propria (pT2), and 22 were muscle invasive (pT3). One hundred twenty were classified as Grade 1, 53 as Grade 2, 54 as Grade 3, with 9 carcinoma in situ. Of the 20 nonrecurrent tumors, 18 were staged and graded as pT1 G1, one tumor as pT1 G1, and one as pT1 G2.

RESULTS

Gene Amplification. Twenty-four of the 256 tumors analyzed were amplified for C-erbB-2 gene amplification (9%), and this represented 16 of 89 patients with recurrent disease. No C-erbB-2 gene amplification was seen in the nonrecurrent group or the normal controls. Gene copy number did not exceed 8 (when compared with $N87$) >8 copies.

Table 2: Sensitivity of competitive PCR for C-erbB-2 copy number

<table>
<thead>
<tr>
<th>Assay</th>
<th>Lower limit</th>
<th>Mean (±2 SD)</th>
<th>Upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 using $^{32}$P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT20$^a$</td>
<td>0.828 ± 0.016</td>
<td>1.16</td>
<td>1.65</td>
</tr>
<tr>
<td>MDA453$^b$</td>
<td>1.169 ± 0.148</td>
<td>1.467</td>
<td></td>
</tr>
<tr>
<td>SKBR3$^d$</td>
<td>1.949 ± 0.36</td>
<td>2.69</td>
<td></td>
</tr>
<tr>
<td>Day 1 using $^{32}$P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA453$^c$</td>
<td>0.655 ± 0.118</td>
<td>0.899</td>
<td></td>
</tr>
<tr>
<td>MDA361$^c$</td>
<td>1.106 ± 0.062</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>SKBR3$^b$</td>
<td>1.037 ± 0.2</td>
<td>1.437</td>
<td></td>
</tr>
<tr>
<td>N87$^b$</td>
<td>2.141 ± 0.352</td>
<td>2.845</td>
<td></td>
</tr>
<tr>
<td>Day 2 using $^{32}$P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT20</td>
<td>0.663 ± 0.072</td>
<td>0.809</td>
<td></td>
</tr>
<tr>
<td>MDA453</td>
<td>0.822 ± 0.11</td>
<td>1.042</td>
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<tr>
<td>MDA361</td>
<td>1.14 ± 0.288</td>
<td>1.556</td>
<td></td>
</tr>
<tr>
<td>SKBR3$^e$</td>
<td>1.655 ± 0.35</td>
<td>2.353</td>
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<tr>
<td>N87$^e$</td>
<td>2.783 ± 0.534</td>
<td>3.851</td>
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</tr>
<tr>
<td>Day 3 using $^{32}$P</td>
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<tr>
<td>SKBR3$^f$</td>
<td>1.272 ± 0.418</td>
<td>2.109</td>
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<tr>
<td>N87$^f$</td>
<td>1.489 ± 1.194</td>
<td>3.876</td>
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<tr>
<td>Day 3 using $^{32}$P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT20$^g$</td>
<td>0.788 ± 0.076</td>
<td>0.939</td>
<td></td>
</tr>
<tr>
<td>MDA453</td>
<td>0.930 ± 0.208</td>
<td>1.346</td>
<td></td>
</tr>
<tr>
<td>SKBR3$^g$</td>
<td>2.314 ± 0.36</td>
<td>3.051</td>
<td></td>
</tr>
<tr>
<td>N87$^g$</td>
<td>3.774 ± 0.74</td>
<td>5.254</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ C.I.: confidence interval.
$^b$ One copy.
$^c$ Two copies.
$^d$ Four to eight copies.
$^e$ Two to four copies.
$^f$ (N87) >8 copies.
C-erbB-2 gene amplification had progressive disease, indicating a strong statistical association with gene amplification and disease progression (P < 0.005). C-erbB-2 gene amplification, however, was only detected after muscle invasion had occurred. Some heterogeneity was seen in the cohort of patients with C-erbB-2 gene amplification and progressive disease. Four of 14 patients demonstrating gene amplification and evidence of progressive disease at diagnosis failed to show gene amplification in the initial biopsy; however, gene amplification was seen in subsequent biopsies. One patient with superficial disease at presentation and subsequent clinical evidence of extravesical tumor spread also failed to demonstrate consistent C-erbB-2 gene amplification in recurrent biopsies (Table 3). Gene amplification was associated with worsening stage of tumors (P < 0.001) and increasing grade of tumors (P < 0.001; Tables 4 and 5).

**Immunohistochemistry.** C-erbB-2 protein overexpression (at least 1+) was observed in 47% of tumors analyzed (120 of 256) and 11% (14 of 120) with 3+ C-erbB-2 protein overexpression. C-erbB-2 protein overexpression for most patients with recurrent disease remained fairly consistent during follow-up biopsies. Forty-five of 89 patients with recurrent disease (50%) demonstrated C-erbB-2 protein overexpression, compared with 9 of 20 patients with nonrecurrent tumors (45%). No C-erbB-2 protein overexpression was demonstrated in the normal bladder controls (Figs. 5, 6, and 7). There appeared to be no significant association with worsening stage of tumors or increasing grade of tumors (Table 6). C-erbB-2 protein overexpression was fairly evenly distributed between the recurrent and nonrecurrent tumors, and no association could be made with tumor progression or recurrence from the patients analyzed.

**Bladder Cancer Death.** Table 7 demonstrates the results of the univariate and multivariate regression analysis in predicting death from bladder cancer. In the univariate model, age, sex, grade, stage, amplification, and tumors with high levels of C-erbB-2 protein overexpression (3+) were each found to have a significant effect. In the final model after adjusting for other factors, all variables were found to have a significant effect.

**Time to Progression.** Univariate analysis for time to progressive disease in the group of 21 patients with initial superficial tumors demonstrated age (P < 0.02; relative hazard, 1.6; 95% confidence interval, 1.1–2.3), stage (P < 0.02; relative hazard, 1.45; 95% confidence interval, 1.08–1.98), and grade (P < 0.001; relative hazard, 1.78; 95% confidence interval, 1.28–2.46), each having separate effects. After adjusting for other factors, only age (P < 0.05; relative hazard, 1.47; 95% confidence interval, 1.0–2.16) and grade (P < 0.01; relative hazard, 1.67; 95% confidence interval, 1.2–2.33) were significant in the final model. No independent prognostic effect for overall C-erbB-2 protein overexpression (1+) was observed in multivariate analysis (P < 0.3; relative hazard, 0.67; 95% confidence interval, 0.36–1.25) or for the subset of 3+ tumors (P < 0.6; relative hazard, 1.28; 95% confidence interval, 0.53–3.10). Gene amplification was not included in the model because increased C-erbB-2 copy number was detected only after progression had occurred. Risk factors predictive of bladder cancer death for those tumors with disease progression are shown in Table 8. Age and grade were the only significant variables in the final model. C-erbB-2 protein overexpression did not appear to be an important prognostic variable in predicting bladder cancer death in univariate and multivariate analysis for these patients.

### Table 3 Association of tumor grade with amplification

<table>
<thead>
<tr>
<th>C-erbB-2 amplification</th>
<th>Totals</th>
<th>AMP</th>
<th>1 copy</th>
</tr>
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<tbody>
<tr>
<td>CIS</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>pTxE</td>
<td>169</td>
<td>10</td>
<td>159</td>
</tr>
<tr>
<td>pTx1</td>
<td>36</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>pT2-4</td>
<td>22</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

- *a* n = 236; P < 0.001.
- *b* CIS, carcinoma in situ; pT1x, tumor involving mucosa only. pT2-4, tumor involving lamina propria but not muscle. pT2-4, tumor involving superficial and deep muscle layers.

### Table 4 Association of tumor stage with amplification

<table>
<thead>
<tr>
<th>C-erbB-2 amplification</th>
<th>Totals</th>
<th>AMP</th>
<th>1 copy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>120</td>
<td>3</td>
<td>117</td>
</tr>
<tr>
<td>Grade 2</td>
<td>53</td>
<td>1</td>
<td>52</td>
</tr>
<tr>
<td>Grade 3</td>
<td>54</td>
<td>20</td>
<td>34</td>
</tr>
</tbody>
</table>

- *a* n = 227; P < 0.001.
- *b* CIS, carcinoma in situ; pT1x, tumor involving mucosa only. pT2-4, tumor involving lamina propria but not muscle. pT2-4, tumor involving superficial and deep muscle layers.

- *c* Amplification (>)1 copy.
- *d* Normal diploid content of cell.
Fig. 5. Immunohistochemical plate demonstrating normal bladder transitional cell mucosa with no C-erbB-2 protein expression.

Fig. 6. Immunohistochemical plate demonstrating C-erbB-2 protein overexpression in an invasive transitional cell carcinoma. Section a was scored 1+ for C-erbB-2 protein overexpression. The control (b) was negative.
Fig. 7. Immunohistochemical plate demonstrating C-erbB-2 protein overexpression in transitional cell carcinoma of the bladder. Section a was scored 3+ for C-erbB-2 protein overexpression. The control (b) was negative.

**Time to Recurrence.** Age ($P < 0.02$; relative hazard, 1.45; 95% confidence interval, 1.02–2.05), stage ($P < 0.02$; relative hazard, 1.45; 95% confidence interval, 1.07–1.96), and grade ($P < 0.01$; relative hazard, 1.57; 95% confidence interval, 1.13–2.18) had significant effects in time to recurrence for univariate analysis. Only grade ($P < 0.05$; relative hazard; 1.46; 95% confidence interval, 1.03–2.06) had an independent effect in the final model. Those patients ($n = 22$) with invasive disease at presentation were not included in the analysis because it was felt that residual disease would have remained after the initial cystoscopy.

**Survival.** Survival for the 54 patients with C-erbB-2 protein overexpression was compared to the 55 patients without C-erbB-2 protein overexpression for the primary tumor. No significant difference was found between the two groups ($\chi^2 = 1.57$, 1 df; $P < 0.25$). Protein overexpression failed to demonstrate any significant difference for survival in the smaller cohort of 21 patients with evidence of progressive disease when the primary tumor was analyzed ($\chi^2 = 2.2$, 1 df; $P < 0.25$). Survival analysis also failed to demonstrate C-erbB-2 gene amplification as a prognostic indicator for a subgroup of more aggressive tumors in those 43 patients with progressive disease ($\chi^2 = 0.98$, 1 df; $P < 0.5$). Median survival for patients with progressive disease showed no significant difference for amplified versus nonamplified tumors ($\chi^2 = 1.12$, 1 df; $P < 0.5$).

**DISCUSSION**

Human bladder cancer is multistage with a typical onset late in life. Its pathogenesis and progression has been related to a number of genetic alterations including activation of oncogenes and inactivation of tumor suppressor genes (29). Bladder cancer provides not only an excellent model to analyze these genetic events but may also provide a much needed molecular marker to identify those patients at risk for tumor recurrence and disease progression.

Gene amplification is an important event in the induction/progression of many tumors. As more data becomes available, the clinical consequences of these genotypic changes become evident. In neuroblastoma, the copy number of the N-myc gene needs to be assessed accurately in order to predict severity of disease and, therefore, treatment response (30, 31). How accurate gene copy number needs to be
tumor behavior. Results presented here fail to show any correlation with C-erbB-2 protein overexpression and worsening stage or increasing grade. Survival was not influenced by overexpression of the C-erbB-2 oncoprotein for the initial tumor, nor did it identify a subgroup of aggressive tumors at the time of disease progression. Univariate and multivariate analysis failed to identify C-erbB-2 oncoprotein overexpression as a prognostic factor for time to recurrence or progression. Stage and grade have been shown to be important determinants of progression and death from bladder cancer. Tumor grade has been found to be of value in predicting future recurrence and progression in univariate analysis of superficial bladder cancer (32–34). This report confirms these findings, and in our final models, tumor grade was found to be of independent prognostic value.

Our data has shown that overexpression parallels, in most cases, gene amplification. Five patients, however, with low gene copy number failed to show protein overexpression. Fluorescent in situ hybridization studies have suggested that this increased copy number may be due to chromosome 17 aneusomy (12). Aneusomy of 17 appears neither to be mandatory nor sufficient for protein overexpression but may represent DNA aneuploidy rather than an independent genetic event. The use of two single copy reference genes in the differential PCR has tried to overcome the problem of increased copy number of C-erbB-2 being due to DNA aneuploidy, and we are in the process of using a single copy reference gene on 17 to determine chromosome copy number on the amplified, nonoverexpressing tumors. Keith et al. (35) demonstrated coamplification of the retinoic acid receptor α, erbB-2, and topoisomerase II α in breast tumors. These genes have been mapped to chromosome 17q21. The coamplification of these genes suggests a physical linkage on chromosome 17q in breast tumors; however, the distinction of aneuploidy from amplification, the limits of amplification, and overexpression of C-erbB-2 protein from these tumors were not determined. This study suggested that amplification of genes linked to C-erbB-2 may provide a molecular marker with which to establish new prognostic markers and subtype a group of tumors with amplified C-erbB-2, which generally have a poor prognosis. Hubbard et al. (36) demonstrated from 323 breast tumors that a substantial number with C-erbB-2 gene amplification lacked detectable protein overexpression. This was shown to illustrate the complex nature of C-erbB-2 gene dysregulation in cancer, and factors acting as promoters or suppressors of gene function may directly affect transcription, regardless of amplification status. Further direct evidence of factors affecting transcription comes from studies of

### Table 6 Association of tumor category, grade, and C-erbB-2 protein overexpression

<table>
<thead>
<tr>
<th>C-erbB-2 protein</th>
<th>CIS n = 9</th>
<th>pTa n = 169</th>
<th>pT1 n = 36</th>
<th>pT2-4 n = 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Grade 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Grade 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Totals</td>
<td>7</td>
<td>2</td>
<td>77</td>
<td>92</td>
</tr>
<tr>
<td>% positive</td>
<td>78%</td>
<td>45%</td>
<td>56%</td>
<td>36%</td>
</tr>
</tbody>
</table>

*a n = 236. CIS, carcinoma in situ; pTa, tumor involving mucosa only; pT1, tumor involving lamina propria but not muscle; pT2-4, tumor involving superficial and deep muscle layers.

*b Grade 1, well-differentiated tumor. Grade 2, moderately differentiated tumor. Grade 3, poorly differentiated tumor. +, At least 1+ C-erbB-2 protein overexpression. −, no protein overexpression.

### Table 7 Multivariate and univariate regression for risk factors predictive of bladder cancer death

The "final model" is the model produced at the end of the backward elimination procedure. For a term included in the final model, P denotes the significance of that term, adjusting for the effects of all the terms that are in the final model.

<table>
<thead>
<tr>
<th>Factor</th>
<th>P</th>
<th>Relative hazard point estimate</th>
<th>95% C.I.</th>
<th>P</th>
<th>Relative hazard point estimate</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt;0.01</td>
<td>1.75 (1.2-2.5)</td>
<td>&lt;0.05</td>
<td>1.53 (1.02-2.29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>&lt;0.05</td>
<td>0.46 (0.44-0.50)</td>
<td>&lt;0.05</td>
<td>0.4 (0.17-0.85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>&lt;0.001</td>
<td>2.09 (1.55-2.82)</td>
<td>&lt;0.001</td>
<td>1.93 (1.29-2.84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>&lt;0.001</td>
<td>2.73 (2.64-2.83)</td>
<td>0.01</td>
<td>1.8 (1.16-2.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>&lt;0.001</td>
<td>3.08 (1.58-5.93)</td>
<td>&lt;0.02</td>
<td>2.45 (1.29-5.41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-erbB-2</td>
<td>1.0</td>
<td>0.86 (0.48-1.54)</td>
<td>&lt;0.02</td>
<td>0.41 (0.2-0.86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-erbB-2 3+</td>
<td>&lt;0.01</td>
<td>2.57 (1.25-5.29)</td>
<td>&lt;0.02</td>
<td>3.04 (1.17-7.46)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a n = 109. C.I., confidence interval.

is open to debate and whether semiquantitative data gives as much prognostic information over more precise methods remains to be shown.

In agreement with other recent publications, C-erbB-2 gene amplification was not found to be a frequent cause of protein overexpression in bladder cancer (12–14). In this series, only 9% of total tumors showed amplification, with 47% demonstrating protein overexpression, suggesting that multiple mechanisms leading to overexpression may coexist within a single tumor. What has been important is to determine at what point in this multistage progressive disease amplification and overexpression occurred and whether they proved to be independent diagnostic and/or prognostic indicators for recurrence and disease progression.

From the clinical data presented, 14 of 16 of the patients with gene amplification had progressive disease. Gene amplification was only detected after disease progression. The prognostic factors for bladder cancer death showed an association in the final model for C-erbB-2 gene amplification, but from the survival data, C-erbB-2 gene amplification did not identify a subset of patients with a more aggressive form of progressive disease. Immunohistochemistry for detecting the C-erbB-2 oncoprotein remains a reliable semiquantitative technique for paraffin-embedded sections. Direct comparison with other studies can sometimes prove difficult due to differences in primary antibody, amplification systems, scoring, and interpretation of these scores. It is clear, however, that overexpression can occur without gene amplification.

It has been suggested that C-erbB-2 protein overexpression, together with other predictive parameters, may serve to provide a phenotypic profile that permits more accurate forecasting of bladder
breast cancer cell lines in which C-erbB-2 protein can be down-regulated by estrogen complexed with its receptor (37). Increased C-erbB-2 mRNA levels resulting from elevated amounts of a transcription factor have also been observed in cancer cells with no detectable gene amplification (38). Other explanations include physical damage to the gene, mutation, or absence of promoters. The present state of knowledge of C-erbB-2 biology is thus complex, and in this study, we have demonstrated a lack of correlation between protein overexpression and disease course. This could suggest that C-erbB-2 gene amplification is a marker for bladder cancer progression by virtue of its coamplification with some other relevant gene.

The prognosis of bladder cancer is closely associated with tumor category, meaning patients with high stage disease have a greater chance of development of metastases or of dying. Our results, in agreement with other studies, confirm that tumor stage and grade are important determinants of death from bladder cancer. Inter- and Intrapathological observer variation has been shown to vary between 15 and 50% (39); therefore, other important indicators to identify those patients at risk will be required and may emerge as the molecular events involved in bladder carcinogenesis unfold. Differential PCR and immunohistochemistry can provide valuable information on the role of oncogenes in the initiation and progression of human cancer. These methods however, do not overcome the problems associated with heterogeneity and stromal contamination when assessing gene copy number. This may explain the relatively low copy numbers of C-erbB-2 gene amplification in this series and also explain why five patients with progressive disease did not show consistent gene amplification in their biopsies. By using archival bladder specimens, we have shown that C-erbB-2 gene amplification appears to be a late event. Despite the small number of patients, these results suggest that the use of C-erbB-2 gene copy number as a diagnostic tool in detecting patients with established muscle-invasive cancer appears to far outweigh its potential as a prognostic marker in predicting which patients will develop progressive, muscle-invasive bladder cancer.

ACKNOWLEDGMENTS

We thank Drs. W. Angerson and J. Love for their statistical advice and D. Campbell for his technical assistance.

REFERENCES


CLEVELAND C-ERBB-2 GENE AMPLIFICATION

2430

C-erbB-2 Gene Amplification: A Molecular Marker in Recurrent Bladder Tumors?

Mark Underwood, John Bartlett, Jonathan Reeves, et al.


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