Identification of Patients with Transitional Cell Carcinoma of the Bladder Overexpressing ErbB2, ErbB3, or Specific ErbB4 Isoforms: Real-Time Reverse Transcription-PCR Analysis in Estimation of ErbB Receptor Status from Cancer Patients

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ABSTRACT

Purpose: The purpose of this research was to quantitatively analyze tumor-specific overexpression of all ErbB receptors and ErbB4 isoforms in transitional cell carcinoma (TCC) of the bladder.

Experimental Design: A real-time reverse transcription-PCR protocol was set up to simultaneously quantitate the mRNA levels of all four of the ErbB receptors and ErbB4 isoforms. Exon-intron structure of the ErbB4 gene was determined for ErbB4 isoform analysis. The assay was validated by analyzing: (a) defined ErbB cDNAs; (b) cell lines transfected with defined ErbB cDNAs; and (c) cancer cell lines with ErbB status controlled by Western blotting. ErbB mRNA expression was quantitated from 29 clinical samples representing TCC, interstitial cystitis, or histologically normal bladder. Cutoff expression levels predicting neoplasia at 95% probability were determined. ErbB expression and amplification was analyzed by immunohistochemistry and chromogenic in situ hybridization.

Results: Experiments with control cDNAs and cell lines demonstrated that the assay was both specific and sensitive, and that ErbB mRNA levels closely correlated with protein levels in cancer cell lines. Determination of cutoff expression levels indicated tumor-specific overexpression of ErbB2, ErbB3, and specific ErbB4 isoforms in a subset of TCC patients. Significant overexpression of ErbB mRNAs was also detected in cases without amplification of the respective gene or when the protein product was not localized at the cell membrane.

Conclusion: Bladder cancer patients with tumor-specific overexpression of ErbB receptors or their isoforms were identified. Real-time reverse transcription-PCR could be used for ErbB receptor status quantitation to produce prognostic and predictive information for cancer therapy.

INTRODUCTION

The ErbB subfamily of receptor tyrosine kinases consists of four homologous proteins: the epidermal growth factor receptor (also named ErbB1 or HER1), ErbB2 (HER2, c-Neu), ErbB3 (HER3), and ErbB4 (HER4; Refs. 1–4). ErbB receptors are frequently overexpressed and/or their respective genes are amplified in human neoplastic tissues. ErbB receptor overexpression has both prognostic and therapeutic significance (5, 6). A humanized antibody against ErbB2 (Herceptin) is now in clinical use for ErbB2-positive metastasized breast cancer (7), and a tyrosine kinase inhibitor of ErbB1 (ZD1839) has been approved for therapy of advanced non-small cell lung cancer. Moreover, several compounds that block ErbB2, ErbB1, or various ErbBs simultaneously are currently being tested in clinical trials for different cancer types. However, preclinical evidence about the expression levels of specific ErbBs in tumor tissues is often controversial. One example of a cancer with contradictory reports about ErbB expression levels, as well as about the prognostic and predictive significance of ErbB expression, is TCC of the bladder.

Cancer of the urinary bladder is the fifth most common cancer in men in western countries, and its incidence is rising (8). Bladder cancers are divided into histologically distinct subtypes of which tumors arising from the transitional epithelium comprise ~90% of patients (9). A role has been suggested for ErbBs both as prognostic indicators and drug targets in TCC.

2 The abbreviations used are: TCC, transitional cell carcinoma; RT-PCR, reverse transcription-PCR; GPS, glutamine/penicillin/streptomycin; IHC, immunohistochemistry; CT, threshold cycle; CISH, chromogenic in situ hybridization; JM, juxtamembrane; CYT, cytoplasmic isoform; FISH, fluorescent in situ hybridization.

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Several laboratories have reported immunohistochemical expression studies of ErbB1 and ErbB2 in TCC with variable results. Conclusions of these reports suggest the presence of ErbB1 in a percentage of TCC samples ranging from 23 to 100% (10–14), or ErbB2 in a percentage ranging from 2 to 74% (15–20). Few attempts to quantify ErbB proteins in TCC and normal tissues have indicated overexpression of ErbB1 (10, 20, 21) or ErbB2 (16, 19, 20) in TCC, and ErbB1 or ErbB2 gene amplification has been reported in 3–5% (12, 22) or in 3–32% (16, 19, 23, 24) of TCC cases, respectively. However, immunohistochemical analyses also suggest a change in the distribution rather than actual overexpression of ErbB1 in TCC (25, 26), or that the expression of both ErbB1 and ErbB2 is in fact down-regulated in TCC when compared with normal urothelium (14). Few studies have also addressed the expression of ErbB3 and ErbB4 in TCC. These results demonstrate immunohistochemical localization of ErbB3 in 20–56% (14, 20, 27) and ErbB4 in 11–30% (14, 27) of cases, but also suggest reduced expression of ErbB3 and ErbB4 in TCC when compared with normal tissues (14). In addition, none of the papers have analyzed expression of specific ErbB4 isoforms (28), which differ significantly from each other in their structure (29, 30), function (30, 31), and distribution in tumors (32).

Given such diverse results about ErbB expression, as well as the shortage of quantitative information, the statistical analyses of ErbB expression patterns and clinical parameters have resulted in variable conclusions about the prognostic significance of the ErbB expression in TCC (14, 19, 20, 27, 33–37).

Currently, drugs blocking the function of either ErbB1 or ErbB2 are being tested in Phase II clinical trials for the treatment of TCC. Hypothetically, to optimize the response rate and to minimize the side effects, these drugs should be targeted to selected patients that express the molecular targets at higher levels than normal tissues (38). However, testing this hypothesis has been hampered by the lack of methods to quantitate the expression of specific ErbB gene products. Here we describe the setting up and validation of a quantitative real-time RT-PCR-based method that can be used to simultaneously quantify the levels of mRNAs encoding all of the ErbB receptors, as well as the specific ErbB4 isoforms. We determined ErbB expression levels from clinical tissue samples of histologically normal bladder, the inflammatory disorder interstitial cystitis, and TCC. Tumor-specific overexpression of ErbB2, ErbB3, as well as ErbB4 isoforms of the JM-a type, was found in a subpopulation of TCC patients. Quantitative information about ErbB status may be critical for selecting patients for ErbB inhibitor therapies, as well as for additionally evaluating the prognostic potential of ErbBs in cancer.

MATERIALS AND METHODS

**cDNA Plasmids.** pEBS7 plasmids with cDNA inserts encoding ErbB1, ErbB2, ErbB3, or ErbB4 isoform JM-a CYT-2 (39) were kindly provided by Dr. Mikala Egeblad (University of California San Francisco, San Francisco CA). pcDNA3.1 plasmids with cDNA inserts encoding each of the four ErbB4 isoforms will be described elsewhere.

**Cell Lines.** Human bladder cancer cell lines 5637 and T24 were obtained from American Type Culture Collection, and cultured in DMEM supplemented with 10% FCS (Sigma) and 1% GPS supplement (Irvine Scientific). T-47D breast cancer cells were from American Type Culture Collection, and cultured in RPMI with 10% FCS and 1% GPS. Wild-type NIH 3T3 clone 7 mouse fibroblasts, as well as NIH 3T3 clone 7 fibroblasts overexpressing ErbB1, ErbB2, ErbB3, or ErbB4 (40), were cultured in DMEM with 10% FCS and 1% GPS. 32D mouse myeloid cells as well as 32D transfectants overexpressing different ErbB4 isoform were cultured in RPMI 1640 with 10% FCS, 1% GPS, and 5% conditioned medium from a culture of interleukin-3-producing WEHI cells.

**Clinical Tissue Samples.** Tissue samples were produced in routine surgical procedures at the university hospitals of Turku and Tampere in Finland during the years 1993–2001. Six samples were collected from histologically normal regions of urinary bladders from patients with prostate cancer. In addition, 5 samples from patients with interstitial cystitis and 18 samples from patients with grade II or III TCC were collected. Part of the samples was frozen in liquid nitrogen for subsequent RNA isolation and another part was processed into paraffin sections for pathological diagnosis and IHC.

**Western Blot.** ErbB protein expression was analyzed from cell lines by Western blotting and enhanced chemiluminescence, as described (31). Primary antibodies used were antireceptors for ErbB1, C-18 for ErbB2, C-17 for ErbB3, and C-18 for ErbB4 (all from Santa Cruz Biotechnology). A peroxidase-conjugated goat antirabbit IgG antibody (Jackson Immuno-research Laboratories) was used as a secondary antibody.

**RNA Isolation and cDNA Synthesis.** Total RNA was extracted from cultured cells using RNAzol B reagent (Tel-Test Inc.) according to the manufacturer’s protocol. From clinical tissue samples RNA was extracted using Qiagen RNeasy Mini kit (Qiagen Inc.) or as described (41, 42). To eliminate possible contaminating DNA, RNA samples were treated with 5 units of DNase I (Roche).

cDNA was synthesized in a reaction containing 1 µg of total RNA as a template, 0.5 µg random hexamers (Promega), and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega), in a total volume of 25 µl according to the manufacturer’s protocol. Duplicate reactions were done both in the presence and absence of the reverse transcriptase enzyme to control for false positive amplification resulting from contaminating chromosomal DNA.

**Real-Time PCR.** Real-time PCR analysis of cDNA samples was performed with specific primers and fluorescent probes designed using Primer Express software (PE Biosystems). To specifically quantitate the ErbB4 isoforms, probes for JM-a, JM-b, CYT-1, or CYT-2 were designed to anneal to exons 15, 16, 25, and 26, or the junction between exons 25 and 27, respectively (Fig. 1; Table 1). The primers were synthesized focused on exon sequences flanking the site recognized by the probe (Fig. 1; Table 1). Primers and probes for ErbB1, ErbB2, and ErbB3 were designed to similar unconserved regions as the reagents de-
ErbB Overexpression in Bladder Cancer

Fig. 1 Schematic representation of the ErbB4 mRNA and partial exon structure of the alternatively spliced receptor isoforms. Fluorescent oligonucleotide probes (horizontal bars), which were used in real-time RT-PCR analysis to differentiate ErbB4 isoforms, were designed to hybridize to isom-form-specific exons 16, 15, and 26 to detect isoforms JM-a, JM-b, and CYT-1, respectively, or to the junction between exons 25 and 27 to detect isoform CYT-2. FOR, forward primer-5'-primer; REV, reverse primer-3'-primer; B, isoform CYT-1; IV, extracellular domains I-IV; TM, transmembrane domain; TK, tyrosine kinase domain; NTR, nontranslated region.

Table 1 Sequences of ErbB-specific primers and TaqMan probes

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Primer/probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ErbB1</td>
<td>5'-primer</td>
<td>5'-CCACCTGTGGCCATCCAAAATC-3'</td>
</tr>
<tr>
<td></td>
<td>3'-primer</td>
<td>5'-GGCGATGGACGGGATATT-3'</td>
</tr>
<tr>
<td></td>
<td>probea</td>
<td>5'-CCGACTCTGTTGACGTGAAT-3'</td>
</tr>
<tr>
<td>ErbB2</td>
<td>5'-primer</td>
<td>5'-GAAGTGGCCCATCCAAAATC-3'</td>
</tr>
<tr>
<td></td>
<td>3'-primer</td>
<td>5'-AACCTTGCCACGTGATAACAT-3'</td>
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<tr>
<td></td>
<td>probea</td>
<td>5'-GTGACTGTCATGTCAGTTG-3'</td>
</tr>
<tr>
<td>ErbB3</td>
<td>5'-primer</td>
<td>5'-CCCTGGACATGGAGCCGATC-3'</td>
</tr>
<tr>
<td></td>
<td>3'-primer</td>
<td>5'-TCACCTGTCAGAAATCAGTTG-3'</td>
</tr>
<tr>
<td></td>
<td>probea</td>
<td>5'-ATGGACGGGCAATTTCCAATCC-3'</td>
</tr>
<tr>
<td>ErbB4 JM-a</td>
<td>5'-primer</td>
<td>5'-CCACCAATGCCCATCCAAA-3'</td>
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<tr>
<td></td>
<td>3'-primer</td>
<td>5'-CCAATTACCTTCGCTGAAATC-3'</td>
</tr>
<tr>
<td></td>
<td>probea</td>
<td>5'-ATGGACGGGCAATTTCCAATCC-3'</td>
</tr>
<tr>
<td>ErbB4 JM-b</td>
<td>5'-primer</td>
<td>5'-CCACCAATGCCCATCCAAA-3'</td>
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<tr>
<td></td>
<td>3'-primer</td>
<td>5'-CCAATTACCTTCGCTGAAATC-3'</td>
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<tr>
<td></td>
<td>probea</td>
<td>5'-ATGGACGGGCAATTTCCAATCC-3'</td>
</tr>
<tr>
<td>ErbB4 CYT-1</td>
<td>5'-primer</td>
<td>5'-CCACATCCCACCTTCCATGCTATAC-3'</td>
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<tr>
<td></td>
<td>3'-primer</td>
<td>5'-ACACTCCTTGGACGACGAAACA-3'</td>
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<td></td>
<td>probea</td>
<td>5'-TGAAATTGGACAGCAGGCCCCCCCT-3'</td>
</tr>
<tr>
<td>ErbB4 CYT-2</td>
<td>5'-primer</td>
<td>5'-CAATCTCCCATCTATAC-3'</td>
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<td>3'-primer</td>
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<tr>
<td></td>
<td>3'-primer</td>
<td>5'-TGACCAAGCTGCATGGACGAC-3'</td>
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*a* Probes were labelled with fluorescent reporter dyes FAM (all ErbB probes) or VIC (Actin probe).

Signed to detect the JM isoforms of ErbB4 (Table 1). To obtain an internal control for experimentation, probes and primers annealing to β-actin mRNA were also synthesized (Table 1). Glycerinaldehyde 3-phosphate dehydrogenase was used as another internal control with similar results to those obtained using β-actin (Pearson correlation coefficient 0.73; *P* < 0.001; *n* = 26).

PCR was carried out in a solution containing 300 nM of primers (Medprobe), 200 nM (except 50 nM for CYT-2 probe) of 5'-6-FAM- or VIC-labeled probe (PE Biosystems), 12.5 μl of TaqMan universal PCR Master Mix (PE Biosystems), and 0.5 μl of template cDNA in a final volume of 25 μl. Thermal cycling was performed with ABI PRISM 7700 Sequence Detector (PE Biosystems). Cycling was initiated with 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C (63°C when ErbB4 CYT-2 was analyzed).

Accumulation of the specific PCR products was detected real-time as an increase in fluorescence. Observed fluorescence was plotted against cycle number to generate amplification plots and to determine C_T values, *i.e.* the cycle numbers at which the fluorescence signal exceeded a 5% of the mean in all of the measurements. Relative expression of the gene analyzed (target gene) was estimated using the formula: relative expression = 2^(-ΔC_T), where ΔC_T = C_T(target gene) - C_T(β-actin). The quantity of ErbB mRNAs was expressed as percentage of the quantity of β-actin mRNA after multiplying relative target gene expression by a factor of 100.
IHC. Five-μm thick formalin-fixed paraffin sections were deparaffinized in Histo-Clear (National Diagnostic) before rehydration. Endogenous peroxidase activity was blocked by a 30-min incubation in 3% H₂O₂ in methanol, and antigen unmasking was performed by two 5-min incubations at 80 °C in 10 mM trisodium citrate (pH 6.0). Nonspecific binding was blocked by overnight incubation at 4 °C with 10% goat serum in PBS. Sections were then incubated for 1 h at 4 °C with primary antibodies. Primary antibodies used were monoclonal NCL-CB11 (1:100 dilution; Novocastra) and polyclonal anti-ErbB-4 (C-18; 1:400 dilution; Santa Cruz Biotechnology), for the detection of ErbB2 and ErbB4, respectively. Epitopes of the primary antibodies were localized by immunoperoxidase technique using Vectastain Elite avidin-biotin complex method and 3,3′-diaminobenzidine peroxidase substrate kits (Vector Laboratories), according to the manufacturer’s protocol. Finally, sections were dehydrated, cleared with Histo-Clear, and mounted with Permount (Fisher Scientific). ErbB2 IHC performed in parallel to CISH analysis (see below) was carried out as described (43).

No specific staining was observed when primary antibody was omitted from the protocol. Specificity of the immunostaining was additionally controlled by simultaneous staining of transfected cell lines with known ErbB expression patterns. To this end, cells were cultured on coverslips, fixed for 15 min at room temperature in 2% paraformaldehyde, and permeabilized for 5 min at 4°C in 0.2% Triton X-100. After the permeabilization step, staining was carried out as described above.

CISH. Genomic amplification of ErbB2 was detected by CISH using a digoxigenin-labeled genomic probe for ErbB2 as described previously (43).

Statistical Methods. Logistic regression analysis was used to investigate the association between the probability of having cancer and the ErbB mRNA expression levels. The aim was to determine cutoff points for different ErbB mRNAs between the non-neoplastic group (patients with histologically normal bladder or interstitial cystitis) and the TCC group. The binary response variable in the analysis was group (no neoplasia or TCC), and ErbB mRNA was a continuous explanatory variable. A second order polynomial model was used in the analysis because the probability of having cancer was high for both very low and high ErbB mRNA. The determination of cutoff points for ErbB mRNAs was based on 95% predicted probability of TCC. Statistical computations were performed using SAS System for Windows, release 8.2.

RESULTS
Exon-Intron Structure of Human ErbB4 Gene. Information about the genomic structures of ErbB receptors was needed to design primers and probes for RT-PCR analysis. However, sequence data about ErbB4 isoforms has only been reported from cDNA clones (31) or from genomic PCR analysis of a short extracellular JM region (44). Therefore, exon-intron structure of the ErbB4 gene was determined by comparing
cDNA sequences of ErbB4 isoforms (4, 29, 30) to human genomic sequences available elsewhere (Fig. 1). The analysis demonstrated that the isoform-specific sequence within the extracellular domain of the JM-a was encoded by exon 16, and its alternative sequence within JM-b, by exon 15. The sequence unique for the CYT-1 was encoded by exon 26, and this exon sequence was lacking from the cDNA of CYT-2 that corresponded to a direct fusion of exon 25 to exon 27 (Fig. 1). The identification of alternative sequences within genomic domains flanked by consensus splice donor and acceptor sites data (not shown) indicates that all of the ErbB4 isoforms are generated by alternative splicing of exons and suggests that there may be isoform-specific regulation of expression.

**Efficiency of the ErbB-specific Primers and Probes.** For real-time RT-PCR, two specific PCR primers, as well as a fluorogenic oligonucleotide probe that anneals to the amplified PCR product were designed (see “Materials and Methods”; Table 1; Fig. 1). To control for the amplification efficiency of the primer/probe sets under defined conditions, real-time PCR protocol was first tested by omitting the RNA isolation and cDNA synthesis protocol, and using plasmids containing purified ErbB cDNAs as templates. Analysis of 1 pg of pEBS vectors with inserts encoding full-length ErbB1, ErbB2, ErbB3, or ErbB4 produced uniform amplification curves that exceeded the given threshold fluorescence value (0.05 relative units) at similar cycle numbers (Fig. 2A). This C<sub>T</sub> number was 22.57, 23.55, 24.66, and 21.71, for ErbB1, ErbB2, ErbB3, and ErbB4 (JM-a) cDNA, respectively. Similar amplification curves as well as C<sub>T</sub> values within a similar range were also obtained when primer/probe sets designed to quantitate different ErbB4 isoforms were tested (Fig. 2B). Real-time PCR with 1 pg of pcDNA3.1 vectors including inserts encoding ErbB4 JM-a CYT-1 or ErbB4 JM-b CYT-2 produced C<sub>T</sub> values of 20.79, 22.10, 20.10, and 20.83 for the variant ErbB4 domains JM-a, JM-b, CYT-1, and CYT-2, respectively. All of the amplification curves were in their linear phases when reaching the threshold fluorescence value, justifying the use of C<sub>T</sub> as a measure of template quantity. No non-specific amplification of an inappropriate template was detected when all of the different ErbB cDNAs were cross-analyzed with all of the primer/probe sets (data not shown). To more directly control for the linearity of association between obtained C<sub>T</sub> values and the concentration of the template in the original samples, standard curves were generated for each primer/probe set by performing the analysis for dilution series of cDNA templates synthesized from purified total RNA samples (Fig. 2, C and D). In these experiments, the coefficients of determination (R<sup>2</sup>) were >0.98 for each of the primer/probe sets (mean = 0.99; SD = 0.008) indicating nearly perfect linearity. These data demonstrate that all of the designed primer/probe sets can be used for quantitative analysis and that the C<sub>T</sub> values obtained from similar quantities of different ErbB cDNAs are comparable.

**Real-Time RT-PCR Analysis of ErbB Expression Is Sensitive, Specific, and Reproducible.** To confirm the specificity of the primers and probes in analysis of purified RNA, real-time RT-PCR analysis was performed for transfected cell lines with defined ErbB expression patterns. Total RNA was isolated from four different cell lines and subjected to cDNA synthesis primed with random oligonucleotides. In addition to specific ErbB signals, the signal for δ-actin mRNA was measured simultaneously from parallel samples, and the observed ErbB expression levels were normalized with δ-actin mRNA expression levels to control for equal efficiency in the RNA isolation and cDNA synthesis steps. Expression of specific ErbB mRNA species was solely detected in cells overexpressing the ErbB subtype matching the primer/probe set used (Fig. 3). As expected, signals with both JM-b and CYT-1-specific reagents were observed from cells overexpressing the ErbB4 isoform JM-b CYT-1 (Fig. 3). Mouse 32D transfectants overexpressing human ErbB4 isoforms with each of the four possible combinations of JM and CYT domains were also subjected to real-time RT-PCR. Signal was again detected only when primers and probes matching the ErbB4 isoforms present in the cells were used (Fig. 4). The interassay variability was tested by repeating the analysis of unselected tumor samples three to five times by two independent investigators. The coefficient of variability in these experiments was maximally 4% (n = 33). These data demonstrate that the real-time RT-PCR analysis is specific, selective, and reproducible.

**ErbB mRNA Levels Measured by Real-Time RT-PCR Correlate with Protein Levels in TCC Cell Lines.** To assess whether ErbB mRNA levels quantitated by real-time RT-PCR reflect the amount of ErbB proteins made by tumor cells, TCC cell lines 5637 and T24 representing grade 2 and grade 3 tumors, respectively, were analyzed by both real-time RT-PCR and Western blotting using ErbB subtype-specific reagents. Both cell lines expressed relatively high levels of ErbB1 and ErbB2 mRNAs ranging from 1.3% to 6.1% of δ-actin mRNA levels (Fig. 5, A and B). The expression of both ErbB1 and ErbB2 mRNAs also seemed to be higher in the grade 2 cell line 5637 when compared with the grade 3 T24 cells, by factors of 4.7 and 2.4, respectively. The mRNA expression levels of ErbB3 and ErbB4 were low in both TCC lines when compared with the levels of a breast cancer cell line T-47D, known to express all ErbB subtypes (Ref. 45; Fig. 5, C and D). When protein expression of the same molecules was analyzed by Western blotting of detergent-soluble membrane molecules (Fig. 5, E–H), and the obtained signals were quantitated by densitometric analysis (Fig. 5, I–L), almost identical results were observed. For example, both TCC lines expressed predominantly ErbB1 and ErbB2 proteins, and the grade 2 cell line 5637 expressed more ErbB1 and ErbB2 than the grade 3 cell line T24, by factors of 5.0 and 1.3, respectively. These data suggest that ErbB mRNA levels correlate with ErbB protein levels in tumor cell lines and demonstrate that ErbB mRNA levels present in genetically nonengineered tumor cells can be quantitated with the real-time RT-PCR protocol.

**Overexpression of ErbB2, ErbB3, and ErbB4 Isoforms in a Subset of TCC Patients.** To test the RT-PCR protocol using clinical tissue material, samples were obtained from uri-
nary bladders from 18 patients with histologically confirmed grade 2 or 3 TCC, from 5 patients with interstitial cystitis, and from 6 patients with histologically normal bladders. Real-time RT-PCR analysis of the non-neoplastic samples (normal bladder and interstitial cystitis) demonstrated relatively high mRNA levels of ErbB1 and ErbB2, and relatively low levels of ErbB3 and the ErbB4 isoform JM-a (Fig. 6). The mean mRNA expression in non-neoplastic tissues was 19%, 9%, 1.5%, and 0.04% of β-actin mRNA, for ErbB1, ErbB2, ErbB3, and ErbB4 JM-a, respectively. Similar to the TCC cell lines (Fig. 5), TCC tissues also predominantly expressed ErbB1 and ErbB2 (Fig. 6). The mean expression levels in TCC samples were 18%, 56%, 4.2%, and 0.1% of β-actin mRNA, for ErbB1, ErbB2, ErbB3, and ErbB4 JM-a, respectively, indicating a tendency for up-regulated expression in cancer for all of the receptors except ErbB1. The mean mRNA expression appeared to be up-regulated in TCC also in case of both CYTs. The mean CYT-1 expression was 0.06% of β-actin mRNA in TCC versus 0.02% in non-neoplastic tissues, and the mean CYT-2 expression was 0.08% in TCC versus 0.01% in non-neoplastic tissues (Fig. 7). Virtually no expression of the JM isoform JM-b was detected in any of the samples, indicating that both of the cytoplasmic variants present were JM-a-type within their JM domains.

However, the more apparent result from the analysis of clinical samples was that a distinct subgroup of individuals with TCC demonstrated ErbB2, ErbB3, or ErbB4 mRNA expression clearly above the levels observed in non-neoplastic tissues or in the majority of TCC samples (Figs. 6 and 7). The highest values detected for ErbB2, ErbB3, and ErbB4 (CYT-2 isoform) exceeded the mean of expression signals in the non-neoplastic tissues by 49-fold, 17-fold, and 35-fold, respectively. Because these individuals with particularly high expression would potentially benefit most from ErbB inhibitor therapies, we decided to determine cutoff expression levels for ErbB receptors that could be used to identify TCC patients with exceptionally high ErbB levels. A cutoff mRNA quantity that predicts TCC with 95% probability was chosen and calculated using logistic regression analysis in which all of the non-neoplastic and TCC samples were used. This analysis produced cutoff levels at 28% and 17% of β-actin mRNA for ErbB2 and ErbB3, and cutoff

Fig. 3 Specificity of the real-time RT-PCR method was tested by analyzing RNA extracted from NIH 3T3 cells transfected with cDNAs encoding ErbB1 (A), ErbB2 (B), ErbB3 (C), or ErbB4 isoform JM-b CYT-1 (D). Ranges of values obtained in two parallel analysis were <5% of the means.

Fig. 4 Specificity of the real-time RT-PCR method was tested by analyzing RNA extracted from 32D cells transfected with cDNAs encoding the ErbB4 isoforms JM-a CYT-1 (A), JM-a CYT-2 (B), JM-b CYT-1 (C), or JM-b CYT-2 (D). Ranges of values obtained in two parallel analysis were <5% of the means.
levels at 0.36%, 0.09%, and 0.13% of β-actin mRNA for ErbB4 isoforms JM-a, CYT-1, and CYT-2, respectively. Seven TCC patients (39% of all of the TCC patients) had ErbB2 mRNA levels above the cutoff, and 2 individuals (11%) had ErbB3 mRNA levels above the cutoff (Fig. 6). Two individuals (11%) had ErbB4 values above the cutoff for all three of the ErbB4 isoforms, 4 (22%) for at least two isoforms, and 5 (28%) for at least one isoform (Fig. 7; data not shown). Despite these differences in the numbers of patients exceeding the ErbB4 isoform-specific cutoff values, the same 5 individuals with TCC had the 5 highest ErbB4 mRNA levels with all three of the isoform-specific measurements, suggesting that the expression of isoforms is not regulated independently.

Taken together there were: (a) 2 individuals with expression values that exceeded the cutoff for ErbB2, ErbB3, and ErbB4 (at least for one isoform); (b) 1 individual with values over the cutoff for ErbB2 and ErbB4; (c) 4 individuals with expression values over the cutoff only for ErbB2; and (d) 2 individuals with values over the cutoff only for ErbB4. These 9 individuals together constituted 50% of the analyzed TCC patients. These analyses demonstrate that particular TCC patients overexpress ErbB2, ErbB3, and/or specific ErbB4 isoforms, and that tumor patients with particularly high ErbB expression can be identified by using logistic regression analysis to determine cutoff mRNA levels from clinical samples.

Comparison of Real-Time RT-PCR to IHC in Quantitation of ErbB4 Isoforms. The analysis of clinical samples indicated that ErbB4 isoforms of JM-a-type are expressed in normal bladder tissue and TCC in relatively low quantities (maximally 1% of β-actin mRNA), when compared with ErbB2 (maximally >400% of β-actin mRNA; Fig. 6). However, 22% of TCC patients demonstrated up-regulated mRNA expression that exceeded the set cutoff levels for at least two ErbB4 isoforms (Fig. 7), suggesting that measuring the ErbB4 expression may be biologically relevant. To compare the sensitivity of real-time RT-PCR to IHC, the immunostaining protocol was optimized for both ErbB2 and ErbB4, using cell lines with defined ErbB receptor expression patterns (Fig. 8, C, D, G, and H).
select breast cancer patients with ErbB2 overexpression for Herceptin therapy (38). To compare real-time RT-PCR analysis to ErbB gene amplification analysis, paraffin sections from 5 patients with ErbB2 mRNA levels above the cutoff point were analyzed by CISH analysis. Only 2 of these 5 samples demonstrated amplification of ErbB2 gene. The presence of ErbB2 protein was confirmed by IHC from each sample. An example of a patient without (Fig. 9A) and with (Fig. 9D) ErbB2 gene amplification in the tumor tissue is shown. Both cases had high ErbB2 mRNA (Fig. 9, B and E) and protein (Fig. 9, C and F) expression. These data demonstrate that ErbB2 expression can be up-regulated in TCC both in the absence and presence of ErbB2 gene amplification, and that real-time RT-PCR can be used to identify patients with high expression levels regardless of the amplification status.

**DISCUSSION**

Quantitative information about ErbB expression levels in tumors is necessary, not only for understanding the biological roles of different ErbBs, but also for clinical applications. ErbB expression may correlate with prognosis, or predict responses to chemotherapy, hormonal therapy, or radiation (49, 50). In addition, ErbB inhibitor therapies may produce optimal results if targeted to selected patients with demonstrated overexpression of the receptors. The best available evidence for this is that Herceptin therapy provides clinical benefit almost exclusively to patients with demonstrated ErbB2 overexpression (38). However, commercial assays used to quantitate ErbB2 receptor expression do not always produce consistent results (51, 52), and standardized assays to quantitate ErbB1 (50), ErbB3, or ErbB4 levels are not currently available. Moreover, no method to selectively analyze the expression levels of the functionally different ErbB4 isoforms (28) has been reported.

Here, we describe the setting up and validation of a method based on real-time RT-PCR, designed for quantitation of mRNAs specific for different ErbBs and the ErbB4 isoforms. The quantitative nature of the method was controlled by analyzing dilution series of templates and by demonstrating that the PCR amplification was exponential during the window when the signals were measured. The method was validated by analyses of purified ErbB cDNAs, transfected cells expressing defined ErbBs, and of tumor cell lines with ErbB expression patterns confirmed by Western blotting. Both the specificity and sensitivity of the method were 100% in these experiments. There was no detectable background signal from irrelevant templates and, on the other hand, signal was detected in each case when a relevant template molecule was present in the sample.

The real-time RT-PCR method has several advantages compared with both IHC and FISH, the two assays that are currently most used in clinical assessment of the ErbB receptors status (53). First, real-time RT-PCR is quantitative and provides numeric information that can be used to generate cutoff points, for example, for patient stratification. Both IHC and FISH are semiquantitative at best, and usually quantitated by grading the IHC signal, that are based on
subjective evaluation. Objective quantitation should result in fewer false-negative conclusions, for example, when the subcellular localization of the analyzed molecule is unexpected, as in the case of JM-a isoforms of ErbB4 that may be targeted to nucleus (Fig. 8; Refs. 46, 47). Third, real-time RT-PCR is sensitive. Low, but still statistically significant mRNA expression levels ($\leq 0.001\%$ of $\beta$-actin mRNA) that may be translated to protein levels under the detection threshold of IHC can be reliably quantitated by real-time RT-PCR. In addition, compared with gene amplification analyses, RT-PCR does not produce false-negative results in cases when ErbB protein is overexpressed in the absence of gene amplification. For example, the majority of TCC cases with immunohistochemically demonstrated overexpression of ErbB1 or ErbB2 do not have amplification of the respective genes (12, 17, 18, 22) and, therefore, by FISH analysis would be considered false negatives. These reports are consistent with our finding that only 2 of 5 patients with relative overexpression of ErbB2 mRNA had amplification of the $ErbB2$ gene detected by CISH (Figs. 6 and 9). Finally, the RT-PCR-based method can be used to measure aberrant or alternatively spliced ErbB variants when isoform-specific antibodies are not available. This may turn out to be clinically important in the case of ErbB4 isoforms (28). These naturally occurring isoforms are generated by alternative splicing (Fig. 1).

**Fig. 8** Immunohistochemical localization of the ErbB2 (A and B) and ErbB4 (E and F) in paraffin sections from TCC (A and E) and from histologically normal urinary bladder (B and F). Immunostaining of clone 7 NIH 3T3 cells transfected (C) or not (D) with a plasmid encoding ErbB2 was performed to provide a positive and negative control, respectively, for the anti-ErbB2 antibody. Immunostaining of clone 7 NIH 3T3 cells overexpressing ErbB4 (G) or wild-type clone 7 NIH 3T3 cells (H) was performed to provide a positive and negative control, respectively, for the anti-ErbB4 antibody. Original magnification, $\times 400$.

**Fig. 9** CISH analysis of $ErbB2$ gene amplification status in paraffin sections from two TCC samples (A and D). Real-time RT-PCR analysis was performed from parallel samples from the same 2 patients (B and E). Adjacent sections to those analyzed by CISH were subjected to IHC with an anti-ErbB2 antibody (C and F). Counterstained with hematoxylin. Original magnifications, $\times 200$ (C and F) or $\times 400$ (A and D).
and differ from each other functionally (30, 31). Moreover, our recent findings indicate that some ErbB4 isoforms are more frequently associated with tumor tissues, such as TCC (Fig. 7), breast carcinoma3 and ependymoma (32), than others. Other examples of malignancies from which quantitation of specific ErbB variants could be useful are tumors that overexpress an NH2-terminally truncated form of ErbB1 (54, 55). The ErbB1-specific reagents used in this study were designed to detect both the NH2-terminally truncated as well as the full-length ErbB1.

Disadvantages of the real-time RT-PCR method include problems with the stability of RNA and requirement of frozen tissue samples. However, the quality of RNA as well as the synthesized cDNA can be controlled and standardized by simultaneous measurement of internal control genes, such as β-actin. This requirement for internal controls may indeed lead to better reproducibility compared with IHC that is typically performed without controlling the quality of the samples analyzed (56). In our RT-PCR analyses, the interassay variability was relatively small (coefficient of variability ≤4.0%), demonstrating good reproducibility. When frozen tissue samples are analyzed by PCR-based techniques, there is also no direct visual confirmation about possible differences in tumor cellularity in different regions of the available tumor specimen. Although clearly a disadvantage when compared with IHC, this problem can be partially controlled by careful dissection and histological analysis before processing the tissue specimens for RNA extraction. An important consideration when assessing the value of real-time RT-PCR as a prognostic or predictive assay is also that the method measures ErbB expression at the mRNA level, which does not necessarily correlate with protein expression. On the other hand, data from numerous ErbB expression analyses do not indicate significant imbalance between mRNA and protein levels in tumor cells (32, 57). Also, our comparative analysis of tumor cell lines demonstrated almost perfect correlation between ErbB expression determined by real-time RT-PCR and Western blotting (Fig. 5).

In this study, we analyzed 29 clinical tissue samples from patients with TCC, interstitial cystitis, or histologically normal bladder. The results implicated overexpression of ErbB2, ErbB3, and specific ErbB4 isoforms in individual TCC patients when compared with non-neoplastic samples. Interestingly, relatively high ErbB1 mRNA levels were observed in some patients with interstitial cystitis, as well as in patients with TCC, and no significant tumor-specific overexpression of ErbB1 was found. ErbB mRNA quantities, expressed as percentage of β-actin mRNA content, were used to generate cutoff values that predict malignancy with a 95% probability. Nine of 18 TCC cases (50%) had ErbB2, ErbB3, or ErbB4 levels above this arbitrary cutoff value. These individuals could represent patients who would benefit the most from therapies with specific ErbB inhibitor drugs, such as Herceptin. Large analyses of well-characterized sample series are now needed to optimize the cutoff values to obtain relevant prognostic and predictive information. The clinical value of these selected cutoff points may eventually be assessed in prospective analyses of tumor samples and in clinical trials with ErbB inhibitor drugs. Moreover, similar cutoff values may be determined for several other tumor types with demonstrated ErbB overexpression. No assay to predict, for example, which patients with non-small cell lung cancer respond to ErbB1 inhibitors is currently available (50).

Taken together these results suggest tumor-specific overexpression of ErbB2, ErbB3, and specific ErbB4 isoforms in TCC, which can be reliably quantitated with real-time RT-PCR. Quantitative information about ErbB status could be relevant for prognostics and therapy of a variety of tumors with aberrant ErbB expression patterns.

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REFERENCES


