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Bladder Cancer

Is Quantitative Real-Time RT-PCR an Adjunct to Immunohistochemistry for the Evaluation of ErbB2 Status in Transitional Carcinoma of the Bladder?

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Abstract

Objective: To test different approaches of evaluation of the ErbB2 status in a large series of human transitional cell carcinoma (TCC) of the bladder with the prospect of finding targeted therapies.
Methods: ErbB2 status of 73 human TCC samples was analyzed by both immunohistochemistry (IHC) and by quantification of mRNA levels of expression using real-time reverse transcription-polymerase chain reaction (RT-PCR). Additionally, 18 bladder samples were studied for *ERBB2* gene amplification by real-time quantitative PCR.
Results: Twenty-five tumors (34.2%) overexpressed *ERBB2* mRNA compared to normal bladder samples; this alteration appeared in low-grade and low-stage tumors (pTaG1). Twenty-four (32.9%) tumors showed moderate (++) or strong (+++) immunostaining. A very strong agreement was found between the two methods ($\kappa = 0.97$, 95% confidence interval, 0.90–1). ErbB2 status was not associated with tumor stage. Of the 18 bladder samples tested for *ERBB2* gene amplification, only one showed *ERBB2* DNA amplification.
Conclusions: ErbB2 overexpression occurs in about one third of bladder TCCs. This overexpression can be detected by RT-PCR with a very good correlation with IHC. RT-PCR can therefore be used for cases considered doubtful on IHC rather than gene amplification studies because, in TCC, gene amplification is not the predominant mechanism of both mRNA and protein overexpression. Accurate quantification of ErbB2 status is mandatory for the use of anti-ErbB2–targeted therapies in bladder TCC.

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1. Introduction

The incidence of bladder cancer has increased markedly in recent decades, yet information is still lacking on markers of tumor progression, optimal therapy, and clinical outcome [1,2]. Approximately 90% of malignancies arising in the urothelium are of epithelial origin (transitional cell carcinoma [TCC]). The different outcomes of patients with the same stage and grade tumors call for new molecular markers, which might also serve as therapeutic targets for well-selected patients.

There is an extensive literature on the role of the *ERBB* gene family, particularly *ERBB2*, in various human tumors [3]. ErbB2 overexpression has been well described in breast carcinoma, occurring in approximately 20% of primary invasive tumors and associated with a poor clinical outcome [4]. Studies that have analyzed *ERBB2* gene amplification or ErbB2 protein overexpression in TCC used a variety of techniques and included different study populations. Therefore, their results on the frequency and importance of ErbB2 overexpression and its prognostic significance in TCC remain conflicting [5,6].

In TCC, if protein overexpression varies in the literature from 21% to 89%, gene amplification rarely exceeds 10% of cases (even if the reported range is 4–59%), suggesting cases of overexpression without gene amplification [7]. Rare studies analyzed *ERBB2* by both immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) did not show the ability of FISH to confirm IHC results in TCC.

Here, we quantified *ERBB2* mRNA expression by using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays and protein expression by IHC in 73 bladder TCCs and estimated the agreement between the two methods. We then sought links between the *ERBB2* mRNA and protein expression patterns and pathologic parameters. We also analyzed the relationship between ErbB2 protein or *ERBB2* mRNA overexpression and gene amplification using a very quantitative gene dosage approach: real-time PCR. The study of ErbB2 status by different means is needed for actual or future anti-ErbB2 targeted therapies.

2. Materials and methods

2.1. *Patients and samples*

Specimens of normal bladder tissue from five patients undergoing surgery unrelated to bladder tumors were used as sources of normal bladder DNA and RNA. We analyzed 73 TCC samples from patients managed in two urologic departments who underwent transurethral bladder resection or radical cystectomy between 2001 and 2002. All patients signed an informed consent. Immediately following surgery, tumor samples from each patient were stored in liquid nitrogen for one part of the study (DNA and RNA extraction) and fixed in formaldehyde for the other part (IHC). Classical histologic studies were used for staging and grading of tumor samples according to the TNM 2002 and Mostofi classifications. G2 TCCs were excluded from the study because of their interobserver variability. Each case was independently reviewed by two pathologists (one from each participating center). The patients were 13 women and 60 men with a mean age of 69 yr (range, 42–88 yr). The pathologic stages were: 53 superficial TCC (22 pTa G1, 10 pTa G3, and 21 pT1 G3) and 20 muscle-invasive tumors, all of high grade (≥pT2G3).

2.2. *Real-time RT-PCR*

The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (ie, lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of the gene coding for the TATA box-binding protein (*TBP*; a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and each sample was normalized on the basis of its *TBP* content. We selected the *TBP* gene as an endogenous control because the prevalence of its transcripts is similar to that of the *ERBB2* oncogene and because there are no *TBP* retro-pseudogenes (retro-pseudogenes lead to coamplification of contaminating gDNA and thus interfere with RT-PCR, despite the use of primers in separate exons).

Results, expressed as N-fold differences in *ERBB2* gene expression relative to the *TBP* gene, and termed "RNA N_{ERBB2}," were determined as RNA $N_{ERBB2} = 2^{\Delta Ct}$*sample*, where the ΔCt value of the sample was determined by subtracting the average Ct value of the *ERBB2* gene from the average Ct value of the *TBP* gene.

The RNA N_{ERBB2} values of the samples were subsequently normalized such that the median of the five normal bladder RNA N_{ERBB2} values was 1.

RNA extraction, cDNA synthesis, and PCR conditions are described in detail elsewhere [8]. Briefly, all PCRs were performed using the *Taq*Man PCR Core Reagents Kit (Perkin-Elmer Applied Biosystems, Shelton, CT). Ten microliters of diluted sample cDNA (produced from 2 ng total RNA) was added to 15 μl of the PCR master-mix. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min and 50 cycles at 95 °C for 15 s and 65 °C for 1 min. Experiments were performed with duplicates for each data point. All the patient samples with a coefficient of variation for Ct data >1% were retested.

2.3. *Real-time PCR*

The precise amount of gDNA added to each reaction mix (based on optical density) and its quality (ie, lack of extensive degradation) are both difficult to assess. We therefore also quantified three endogenous DNA control genes, specifically, *ALB* gene mapping to chromosome region (4q11-q13), *SOCS1* (16p13.13), and *THBD* (20p12-cen), and each sample was normalized on the basis of its *ALB* (*or SOCS1 or THBD*) content.