ERBB2 Expression as Potential Risk-Stratification for Early Cystectomy in Patients with pT1 Bladder Cancer and Concomitant Carcinoma in situ

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Abstract

Background/Aims/Objectives: It is difficult to identify patients with a non-muscle-invasive bladder cancer (NMIBC) at stage pT1 with concomitant carcinoma in situ (Cis) who will benefit from an early cystectomy. *Methods:* We retrospectively analyzed clinical data and formalin-fixed paraffin-embedded tissues of patients with NMIBC. Messenger ribonucleic acid (mRNA) expression of progesterone receptor (PGR), estrogen receptor (ESR1), ERBB2, and marker of proliferation Ki-67 (MKI67) was measured by single-step reverse transcription quantitative real-time polymerase chain reaction using RNA-specific TaqMan assays. Relative gene expression was determined by the normalization of 2 reference genes (CALM2, B2M) using the 40 ΔΔCT method and relative gene expression was correlated to the histopathological stage and oncological outcome. *Results:* Of 302 patients with pT1 NMIBC in the initial transurethral resection of the bladder, 65 had a concomitant Cis. Elevated ERBB2 expression (>40.1) significantly correlated with progress in patients with and without concomitant Cis ($p = 0.020$ and $p = 0.049$, respectively). For the subgroup of pT1 with concomitant Cis, elevated ERBB2 expression significantly discriminated between a high-risk group of 55% progression-free survival (PFS) and a low-risk group of 90% PFS after a 5-year follow-up ($p = 0.020$). Cox-regression analysis revealed ERBB2 expression as the only independent prognostic factor for PFS ($p = 0.0037$). *Conclusions:* High mRNA expression of ERBB2 can identify patients with pT1 NMIBC with concomitant Cis, who have a high risk of progression and might benefit from an early cystectomy.

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Introduction

Bladder cancer is the sixth most common malignancy worldwide in male patients and is in the ninth position in terms of both genders [1]. Approximately 75% of the pa-

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tients are diagnosed with non-muscle-invasive bladder cancer (NMIBC) [2]. Depending on the stage and grade after transurethral resection, 50–70% of the patients show disease recurrence and up to 45% of the tumors progress to more invasive stages [3]. Patients with NMIBC are monitored with cystoscopies over many years. Therefore, molecular markers for stratifying patient treatment and application of novel therapeutic drugs are highly appreciated to elucidate the patients' prognosis.

Currently, NMIBC is stratified into 3 risk groups according to clinical (diameter, focality) and pathological (stage, grade, concomitant carcinoma in situ [Cis]) criteria [2]. This is the basis for treatment decision and follow-up strategy. The high-risk group contains a subgroup of highest risk tumors. Among others, the stage pT1 bladder cancer with concomitant Cis is classified as a tumor with the highest risk. In these patients, early radical cystectomy is recommended, if full-dose BCG instillation therapy is refused or if it is not possible to administer the therapy [2]. It has been shown that patients with stage pT1G3 tumors benefit from an early cystectomy [4]. However, while 49% of patients with stage pT1 with concomitant Cis will suffer from progression to muscle-invasive disease [5], half of the patients may be over-treated by radical cystectomy. To date, it is not possible to identify patients in the highest risk group, who will benefit from early cystectomy, which results in a challenging individual treatment decision.

To predict the recurrence and progression of NMIBC, the European Organisation for Research and Treatment of Cancer (EORTC) developed a score (EORTC score) [6]. To improve this limited clinicopathological risk calculator, various molecular markers have been investigated on. Of these, the marker of proliferation Ki-67 (MKI67) has been shown to be associated with the stage and grade in NMIBC [7] and also it has a prognostic impact on NMIBC [8, 9].

More recently, molecular inter-tumor heterogeneity has been investigated leading to the identification of distinct molecular classes beyond histopathological classification resembling the molecular features of the luminal and basal breast cancer subtypes with similar differences in the clinical outcome [10–12]. However, most studies have focused primarily on muscle-invasive bladder cancers (MIBCs) – or on combined analysis of NMIBC and MIBC. MIBC has been shown to be of mainly basal- and luminal-cell origin, reflecting molecular classification of breast cancer [10, 11]. Certain parameters such as HER2 expression can be used to distinguish luminal from basal type bladder cancer. The 4 major determinates of the molecular sub-classification in breast cancer are progesterone receptor (PGR), estrogen receptor 1 (ESR1), ERBB2, and MKI67 [10].

The aim of the present study was to investigate the predictive value of messenger ribonucleic acid (mRNA) expression of the respective markers in the highest risk group of stage pT1 bladder cancer with concomitant Cis compared to stage pT1 without concomitant Cis. To obtain superior sensitivity and objective results, a reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assay, which has been established for breast cancer, was used. This subproject focuses on the relevance of ERBB2 mRNA expression, which revealed to be one of the major determinants of luminal bladder cancer.

Patients and Methods

Study Population

Overall, 302 patients with stage pT1 NMIBC at initial diagnosis who underwent transurethral resection of the bladder between 1989 and 2009 were included. Patients with concomitant Cis (pT1 + Cis) as well as patients without concomitant Cis (pT1 – Cis) were enrolled. Transurethral resection was performed supported by photodynamic diagnosis in most cases since the time Hexvix™ was approved by the FDA and overall, transurethral resection supported by photodynamic diagnosis was performed in about 25% of the patients. Histopathological parameters of all cases, including grading according to World Health Organization (WHO) 1973 and WHO 2004 classification were assessed by a pathologist specialized in uropathology (A.H.). All specimens contained at least 20% tumor cells. All patients underwent re-resection and were treated according to an organ-preserving approach.

Isolation of Tumor RNA

For RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue, a single 10 μm curl was processed according to a commercially available bead-based extraction method (RNXtract® kit; BioNTech Diagnostics GmbH, Mainz, Germany). In brief, a lysis buffer was used to liquefy FFPE tissue slices, while the melting of paraffin was carried out in a thermo-mixer. Tissue lysis was accomplished with a proteinase K solution. Thereafter, lysates were admixed with germanium-coated magnetic particles in the presence of special buffers, which promote the binding of nucleic acids. Purification was carried out by means of consecutive cycles of mixing, magnetization, centrifugation, and removal of contaminants. RNA was eluated with 100 μL elution buffer and RNA eluates were then stored at –80°C until use.

Gene Expression by RT-qPCR

The MammaTyper® is a molecular in vitro diagnostic tool used in the assessment of the gene expression levels of the 4 cancer biomarkers that are required for the clinical management of breast cancer patients in daily routine clinical practice. Instead of using IHC to assess protein expression of HER2, estrogen receptor α, PR, and Ki-67, with MammaTyper®, it is possible to measure the mRNA transcripts of the corresponding genes (ERBB2, ESR1, PGR, and MKI67); this was done by using routine FFPE material and by achieving accurate, reproducible, and objective results.

The mRNA expression levels of ERBB2, ESR1, PGR, and MKI67 as well as of 2 reference genes (REF), namely, B2M and CALM2, were determined by RT-qPCR, which involves reverse transcription of RNA and subsequent amplification of cDNA executed successively as a 1-step reaction. In MammaTyper®, the 6 assays (assay = primer pair and probe specific for the respective target sequence) are duplexed into 3 assay mixes, each using a pair of hydrolysis probes labeled with different fluorophores for separately detecting the duplexed assays.

Each patient sample or control was analyzed with each assay mix in triplicates. The experiments were run on a Light Cycler LC480 II (Roche, CH) based on the following protocol: 5 min at 50°C, 20 s at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and according to MammaTyper® instructions for use 150528-90020-EU Rev. 3.0.

Forty amplification cycles were applied and the cycle quantification (Cq) threshold values of MKI67 and the 2 REF genes for each sample (S) were estimated as the median of the triplicate measurements. These were then normalized against the mean expression of the REF genes and set off against a calibrator (PC) to correct for inter-run variations (ΔΔCq method). The final values were generated by subtracting ΔΔCq from the total number of cycles to ensure that normalized gene expression obtained by the test is proportional to the corresponding mRNA expression levels, a method that facilitates the interpretation of data and clinicopathological correlations. The various calculation steps are summarized in the following formula exemplary for MKI67 (S = sample, pc = positive control):

$$40 - \Delta\Delta Cq(MKI67)S = 40 - \{[Cq(MKI67)S - \text{mean } Cq(REF)S] - [Cq(MKI67)pc - \text{mean } Cq(REF)pc]\}$$

Statistical Methods
After the exclusion of invalid samples from the total cohort on the basis of predefined criteria, pT1 + Cis and pT1 – Cis samples were analyzed and results of the total cohort were correlated to clinical and pathological parameters. The Spearman product-moment correlation coefficient r was used as a measure of the strength and direction of the linear relationship between variables. Statistical analysis including partitioning testing as well as survival analysis based on Kaplan–Meier and Cox regression analysis was performed with JMP SAS (SAS Institute, Cary, NC, USA) and GraphPad Prism software (version 5.04; GraphPad Software Inc., La Jolla, CA, USA).

Results

Patient Population
The total study cohort consisted of 302 NMIBC tumor samples staged pT1 after the central pathological review was completed. Based on predefined criteria, 255 pT1 samples qualified to be analyzed with valid measurements for all 4 biomarkers. In the final cohort of 255 pT1 samples, 65 had a concomitant Cis (pT1 + Cis; Fig. 1). The invalid measurements in 47 samples were due to insufficient RNA input according to prespecified criteria of the
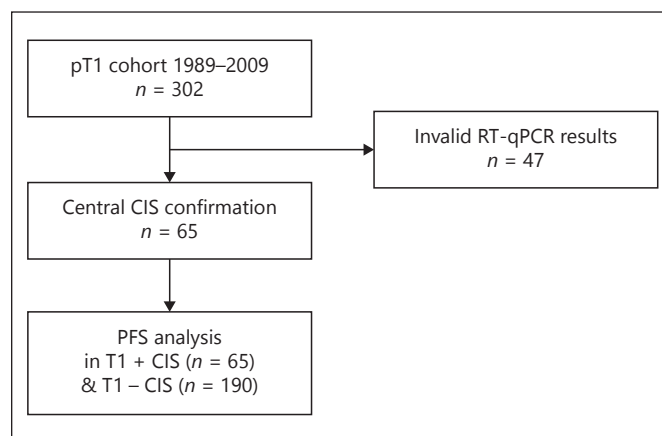

Fig. 1. Remark diagram.

MammaTyper kit and described in the instruction manual of the IVD kit in more detail. In brief, the Cq values of the REF have to be above a minimally required amount of RNA when compared to the synthetic standard quantified in parallel to give a valid sample measurement. Tumor samples were collected from a single institution. The clinicopathological parameters in both cohorts were mostly comparable; only multifocal tumors (29.2% pT1 + Cis vs. 17.4% pT1 – Cis, $p = 0.033$) and grade 3 tumors after WHO 1973 classification (90.8 vs. 66.3%, $p < 0.001$) showed statistically significant differences between the 2 groups (Table 1). The quantitative assessment of ERBB2, ESR1, PGR, and MKI67 mRNA expression resulted in normal distributions with a median expression of 39.95, 35.28, 33.53, and 37.64 in the Cis cohort and 39.98, 34.42, 32.88, and 37.53 in the non-Cis cohort after normalization to the housekeeping genes CALM2 and B2M (Fig. 2).

Correlation between Cis and ESR1, PGR, ERBB2, and MKI67 Expression
ERBB2 mRNA levels were positively correlated to proliferation as determined by MKI67 mRNA quantitation (Spearman rho 0.49; $p < 0.0001$ for pT1 + Cis and Spearman rho 0.27; $p = 0.0002$ for pT1 – Cis; online suppl. Fig. 1, see www.karger.com/doi/10.1159/000453670). In contrast, mRNA levels of the hormone receptors ESR1 and PGR were not positively associated with proliferative activity but rather negatively associated with MKI67. Importantly, both hormone receptors ESR1 and PGR were positively associated with the presence of Cis (Spearman rho 0.19; $p = 0.0025$ and Spearman rho 0.16; $p = 0.0111$, respectively), whereas there was no association between Cis and ERBB2 or MKI67 mRNA expression.

Table 1. Characteristics of bladder cancer patients staged pT1 with and without concomitant Cis

Patient characteristics	Stage pT1 – Cis patients, n (%)	Stage pT1 + Cis patients, n (%)	p value	Total cohort, n (%)
Patient number included	190	65		255
Age ≤75 years	120 (63.2)	46 (70.8)	0.169	166 (65.1)
PDD guided TURB	49 (25.8)	15 (23.1)	0.399	64 (25.1)
Multifocal tumor	**33 (17.4)**	**19 (29.2)**	**0.033**	**52 (20.4)**
Tumor size >3 cm	106 (55.8)	43 (66.2)	0.093	149 (58.4)
WHO grading 1973 G2	**64 (33.7)**	**6 (9.2)**		**70 (27.5)**
WHO grading 1973 G3	**126 (66.3)**	**59 (90.8)**	**<0.001**	**185 (72.5)**
WHO grading 2004 lg	5 (2.6)	0		5 (2.0)
WHO grading 1973 hg	185 (97.4)	65 (100)	0.227	250 (98.0)

Statistically significant results in bold letters.

Cis, carcinoma in situ; hg, high grade; lg, low grade; PDD, photodynamic diagnostic; TURB, transurethral resection of the bladder; WHO, World Health Organization.

Prognostic Value of ERBB2 Expression

Intermediate to high ERBB2 mRNA expression (>40.1) was highly significant concerning progression in the pT1 + Cis cohort ($p = 0.020$) as well as in the pT1 – Cis cohort ($p = 0.049$). As this cut-off value was close to the median expression of ERBB2 mRNA at ~40.0, subgroups of significant size could be separated into high- and low-risk groups (Fig. 3b, c). For the pT1 + Cis cohort, high ERBB2 expression significantly discriminated between a high-risk group of 55% progression-free survival (PFS) after 5-year follow-up and a low-risk group of 90% PFS after 5-year follow-up.

To further explore the prognostic significance of dichotomization into luminal and basal subtypes by using ERBB2, mRNA Cox regression analysis was performed. ERBB2 remained independently significant for PFS $p = 0.0037$; Table 2), when adjusting for gender, tumor size, grading, concomitant Cis and focality, while none of the other clinicopathological factors retained significance.

Discussion

NMIBC still remains a challenging entity for the treating urologist. Until now, stratification of patients into low-, intermediate- and high-risk groups is crucial for further treatment and follow-up. The accepted parameters for classifying NMIBC into these risk groups are pathological staging, grading, number, and diameter of the tumor as outlined in the EAU guidelines [2]. To be able to predict tumor recurrence and progression effectively, the EORTC risk calculator was developed [6].

Here, a concomitant Cis displays the highest single predictive factor for progression, which results in additional 6 points in the risk calculator [6]. This is also recommended in the EAU guidelines, as a pT1 associated with a Cis is subdivided in a subgroup of "highest risk" within the high-risk group [2]. For these patients, an early radical cystectomy should be considered, if intravesical treatment with BCG is refused. Compared to a primary Cis, a concomitant Cis has a worse prognosis [5, 13]. Griffiths et al. [5] reported a 5-year progression rate of 49% in patients with pT1 with associated Cis. Only 30% in this group lived with an intact bladder after 5 years [5]. Patients, who responded to BCG treatment, had a delayed time to progression but without improving the CSS. Thus, the authors of this study do not recommend the intravesical treatment in these highest risk patients [5]. In addition to the pathological features, for T1 with concurrent Cis, no other reliable prognostic markers exist till date.

In this study, we investigated the role of hormone receptor expression of PGR, ESR1, expression of ERBB2 and MKI67, which are all well-established markers in predicting the prognosis of breast cancer and in treatment decision [14]. Spearman's correlation revealed no association between a concurrent Cis and expression of ERBB2 and MKI67. But there was a significant association between ERBB2 and MKI67 mRNA expression, which can also be found in breast cancer [15].

As described earlier, half of all patients with a pT1 with concomitant Cis will show a progress and only a minority will retain an intact bladder, as early radical cystectomy should be considered [5]. By now, it is not
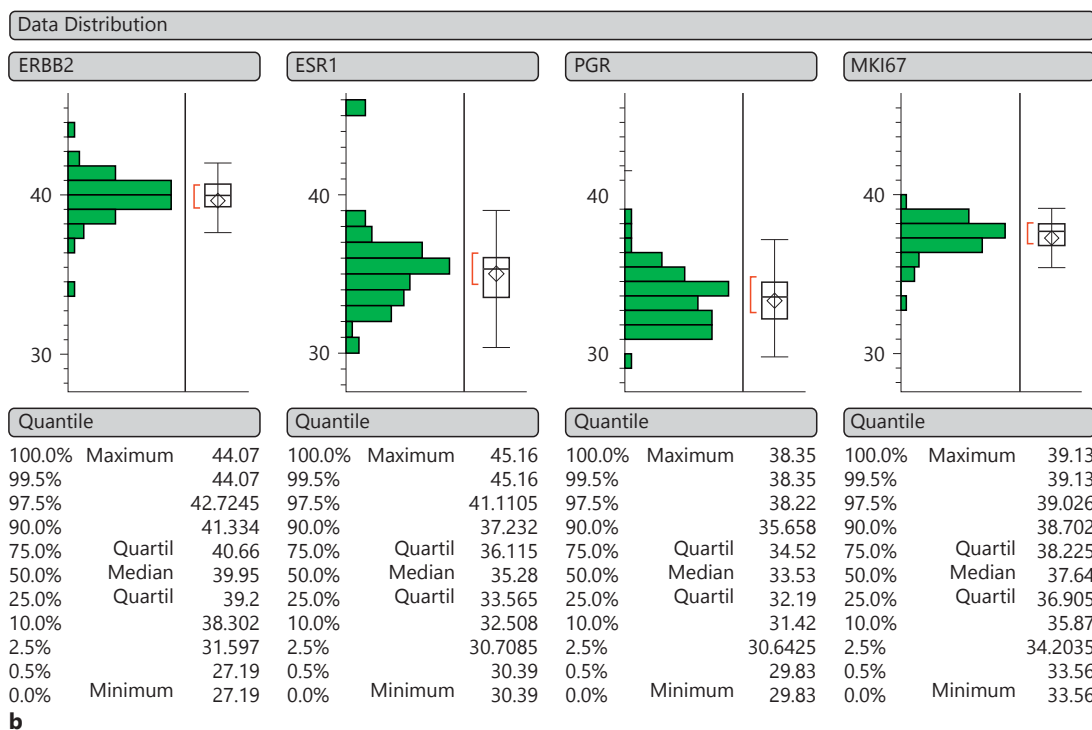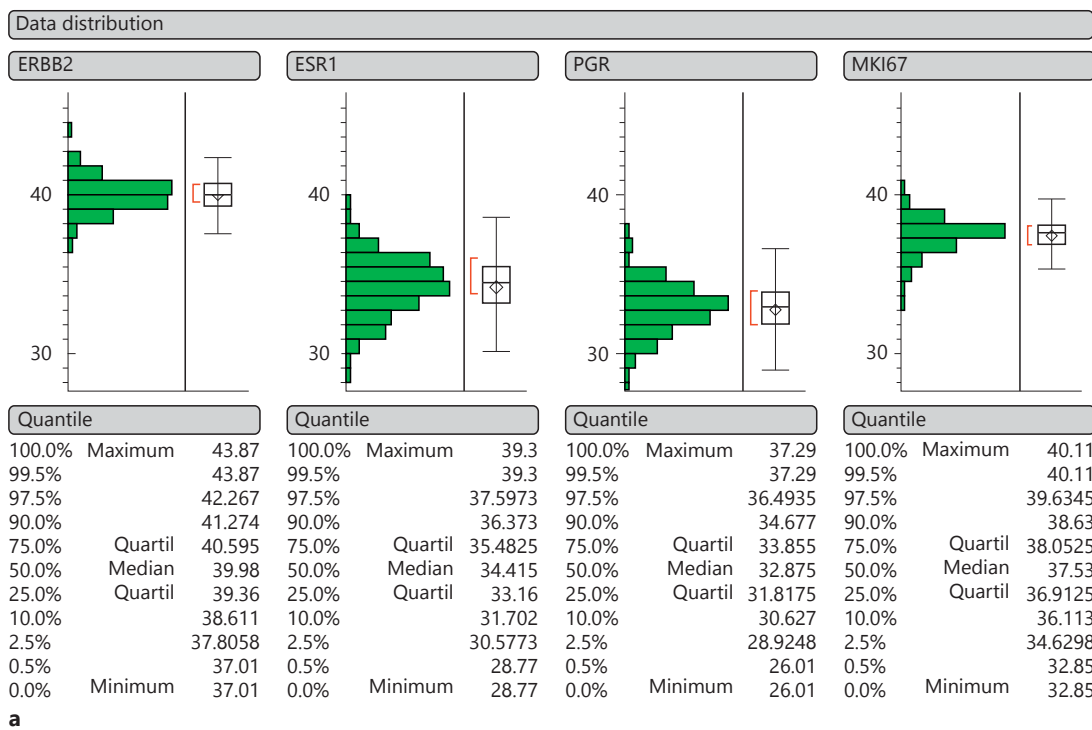
Data distribution

| ERBB2 | ESR1 | PGR | MKI67 |

Quantile (ERBB2)

100.0%	Maximum	43.87
99.5%		43.87
97.5%		42.267
90.0%		41.274
75.0%	Quartil	40.595
50.0%	Median	39.98
25.0%	Quartil	39.36
10.0%		38.611
2.5%		37.8058
0.5%		37.01
0.0%	Minimum	37.01

Quantile (ESR1)

100.0%	Maximum	39.3
99.5%		39.3
97.5%		37.5973
90.0%		36.373
75.0%	Quartil	35.4825
50.0%	Median	34.415
25.0%	Quartil	33.16
10.0%		31.702
2.5%		30.5773
0.5%		28.77
0.0%	Minimum	28.77

Quantile (PGR)

100.0%	Maximum	37.29
99.5%		37.29
97.5%		36.4935
90.0%		34.677
75.0%	Quartil	33.855
50.0%	Median	32.875
25.0%	Quartil	31.8175
10.0%		30.627
2.5%		28.9248
0.5%		26.01
0.0%	Minimum	26.01

Quantile (MKI67)

100.0%	Maximum	40.11
99.5%		40.11
97.5%		39.6345
90.0%		38.63
75.0%	Quartil	38.0525
50.0%	Median	37.53
25.0%	Quartil	36.9125
10.0%		36.113
2.5%		34.6298
0.5%		32.85
0.0%	Minimum	32.85

a

Data Distribution

| ERBB2 | ESR1 | PGR | MKI67 |

Quantile (ERBB2)

100.0%	Maximum	44.07
99.5%		44.07
97.5%		42.7245
90.0%		41.334
75.0%	Quartil	40.66
50.0%	Median	39.95
25.0%	Quartil	39.2
10.0%		38.302
2.5%		31.597
0.5%		27.19
0.0%	Minimum	27.19

Quantile (ESR1)

100.0%	Maximum	45.16
99.5%		45.16
97.5%		41.1105
90.0%		37.232
75.0%	Quartil	36.115
50.0%	Median	35.28
25.0%	Quartil	33.565
10.0%		32.508
2.5%		30.7085
0.5%		30.39
0.0%	Minimum	30.39

Quantile (PGR)

100.0%	Maximum	38.35
99.5%		38.35
97.5%		38.22
90.0%		35.658
75.0%	Quartil	34.52
50.0%	Median	33.53
25.0%	Quartil	32.19
10.0%		31.42
2.5%		30.6425
0.5%		29.83
0.0%	Minimum	29.83

Quantile (MKI67)

100.0%	Maximum	39.13
99.5%		39.13
97.5%		39.026
90.0%		38.702
75.0%	Quartil	38.225
50.0%	Median	37.64
25.0%	Quartil	36.905
10.0%		35.87
2.5%		34.2035
0.5%		33.56
0.0%	Minimum	33.56

b

Fig. 2. Data distribution of ERBB2, ESR1, PGR, and MKI67 mRNA levels in the pT1 + Cis cohort (**a**) and pT1 − Cis cohort (**b**).
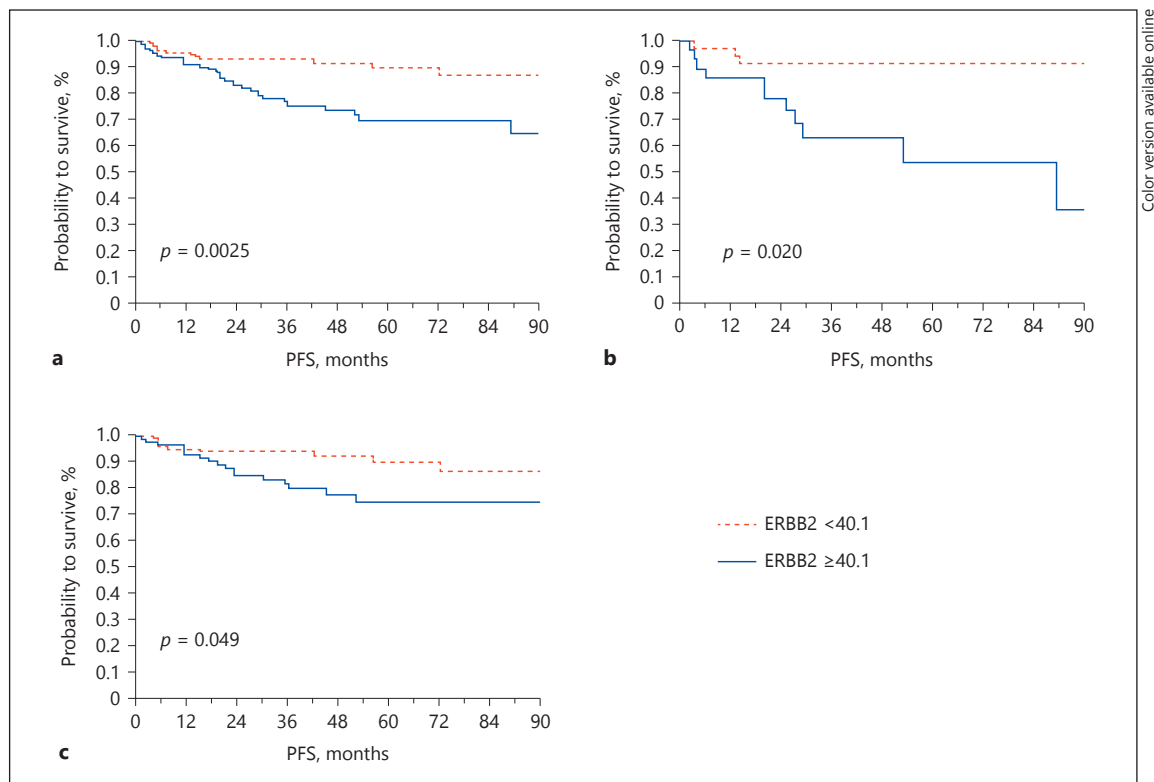
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Fig. 3. Kaplan–Meier analysis of PFS in the total cohort (**a**), pT1 + Cis cohort (**b**), and pT1 − Cis cohort (**c**) based on ERBB2 mRNA expression.

possible to identify those who will face difficulties due to progress and will benefit from early radical cystectomy. In terms of molecular markers, high immunohistochemical expression of Ki67 has been associated with high-grade tumors in T1 [16]. Ki67 expression is furthermore associated with survival in pT1 bladder cancer [8] and also in the subgroup of T1G3 tumors [9]. It has been suggested that Ki67 expression be added to the EORTC risk calculator to improve predictive accuracy of recurrence and progression [17]. In this study, we did not find an association with MKI67 expression in the subgroup of T1 with concomitant Cis. Consequently, it seems not to be suitable for predicting progression in this highest risk group.

In this study, a high expression of ERBB2 could divide the highest-risk group of patients with pT1 and concomitant Cis into a low-risk group with 90% PFS after 5 years from a high-risk group with 55% 5-year PFS. Little is known about the expression of ERBB2 in Cis of the bladder. Gunia et al. [18] could show a strong expression of HER2 in Cis, whereas the non-Cis tissue showed a weak to absent HER2 expression in immunohistochemical stain-

Table 2. Multivariate Cox regression analysis of clinic-pathological parameters and ERBB2 mRNA expression (>40.1) in the total cohort for PFS

	L-R chi^2	p value
ERBB2 (≥40.1 vs. <40.1)	8.44509903	**0.0037**
Gender, female vs. male	0.2523134300	0.6155
Tumor size (>3 vs. ≤3 cm)	1.64541976	0.1996
WHO grading 1973 (G3 vs. G2)	2.50578913	0.1134
Focality (multifocality vs. unifocality)	0.20922888	0.6474
Concomitant Cis (yes vs. no)	2.36918853	0.1238

p values <0.05 are indicated in bold.
Cis, carcinoma in situ; WHO, World Health Organization.

ing. Sjödahl et al. [12] could not find an association between ERBB2 expression and the stage and grade of bladder cancer. Chen et al. [19] could show that ERBB2 amplification could only be found in high-grade NMIBC and goes along with a more aggressive behavior and risk for progression. In this study, the researchers stratified only

for low-risk and high-risk tumors. Furthermore, ERBB2 amplification results in increased ERBB2 mRNA expression in high-grade bladder cancer; this points to a more aggressive behavior of these tumors and this finding is in line with our results [20]. This is underlined by the finding that a strong Her2 expression is found more often in patients with metastatic bladder carcinoma than in those without metastases [21]. In an IHC analysis in pT1G3 NMIBC, HER2 status was not associated with recurrence or progression [22]. The main limitation of IHC-based studies is the lack of standardization and objectiveness. The results for HER2 mRNA expression as well as for the other markers in this study are reproducible and objective and can be easily standardized. Thus, a high HER2 expression may help to identify patients with pT1 and concomitant Cis who will benefit from early cystectomy, as it has been shown in the case of pT1G3 tumors [4].

Furthermore, currently, HER2 also represents a target for a targeted therapy with drugs established in breast cancer. These patients with high expression of HER2 may undergo specific anti-HER2 treatment instead of early cystectomy and instead of BCG therapy. In studies related to bladder cancer cell lines, drugs targeting HER2 showed promising antitumor effects in HER2 overexpressing bladder cancer [23, 24]. The HER2 antibody trastuzumab has been tested in metastatic bladder cancer [25, 26], but to date there is little evidence on the efficacy of this drug.

The major weakness of this study is its retrospective nature with data from a single center. To confirm and verify the results, a validation in a prospective multi-center study will be necessary.

Conclusions

In this study, we could show that high mRNA expression of ERBB2 can divide the highest risk group pT1 with concomitant Cis into a low-risk group for progression and a high-risk group for progression. This has considerable clinical implications, as patients who will benefit from an early cystectomy might be identified at the time of initial diagnosis. This reproducible and objective method should consequently be evaluated in a prospective multicenter study.

Statement of Ethics

Informed consent was obtained from all the individual participants included in the study.

Funding

Disclosure Statement

R.M.W. and S.E. are founders of STRATIFYER Molecular Pathology GmbH. R.M.W. is an employee of STRATIFYER Molecular Pathology GmbH. M.L. and K.S. are employees of BioNTech Diagnostics GmbH.

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