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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

ΔNp63 transcript loss in bladder cancer constitutes an independent molecular predictor of TaT1 patients post-treatment relapse and progression

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Abstract

Purpose Bladder cancer represents a major cause of malignancy-related morbidity and the most expensive *per*-patient-to-treat cancer, due to the lifelong surveillance of the patients. Accurate disease prognosis is essential in establishing personalized treatment decisions; yet optimum tools for precise risk stratification remain a competing task. In the present study, we have performed the complete evaluation of *TP63* clinical significance in improving disease prognosis.

Methods The levels of ΔNp63 and TAp63 transcripts of *TP63* were quantified in 342 bladder tissue specimens of our screening cohort ($n=182$). Hedegaard et al. (Cancer Cell 30:27–42. doi:10.1016/j.ccell.2016.05.004, 2016) ($n=476$) and TCGA provisional ($n=413$) were used as validation cohorts for NMIBC and MIBC, respectively. Survival analysis was performed using recurrence and progression for NMIBC or mortality for MIBC as endpoint events. Bootstrap analysis was performed for internal validation, while decision curve analysis was used for the evaluation of the clinical net benefit on disease prognosis.

Results ΔNp63 was significantly expressed in bladder tissues, and was found to be over-expressed in bladder tumors. Interestingly, reduced ΔNp63 levels were correlated with muscle-invasive disease, high-grade tumors and high-EORTC-risk NMIBC patients. Moreover, ΔNp63 loss was independently associated with higher risk for NMIBC relapse (HR = 2.730; $p=0.007$) and progression (HR = 7.757; $p=0.016$). Hedegaard et al. and TCGA validation cohorts confirmed our findings. Finally, multivariate models combining ΔNp63 loss with established prognostic markers led to a superior clinical benefit for NMIBC prognosis and risk stratification.

Conclusions ΔNp63 loss is associated with adverse outcome of NMIBC resulting in superior prediction of NMIBC early relapse and progression.

Keywords p63 · p53 family · TP63 · TAp63 · Bladder tumors · Urothelial carcinoma

Introduction

Bladder cancer (BlCa) represents the second most common malignancy of the male genitourinary tract, following prostate cancer, and the fourth most frequently diagnosed cancer among male populations in developed countries (Antoni et al. 2017; Torre et al. 2015). Most bladder tumors (90%) originate from bladder urothelium, known as urothelial bladder carcinomas (UBC), whereas the remaining 10% are mostly adenocarcinomas and squamous cell carcinomas (Sanli et al. 2017). The observation that UBC are spatially heterogeneous (Warrick et al. 2019), both in clinical and molecular levels, has led to the establishment of the "*two-pathway model*" for UBC pathogenesis. Characterized by different cell-of-origin and molecular driver events, two

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distinct pathways have been demonstrated to give rise either to non-muscle-invasive bladder cancer (NMIBC; Ta, Tis, T1) or to muscle-invasive bladder cancer (MIBC; T2–T4), approximately 75% and 25% of the newly diagnosed cases, respectively (Castillo-Martin et al. 2010; Kamat et al. 2016; Knowles and Hurst 2015).

Disease-specific mortality has declined recently owing to technological advances of disease therapy and surveillance (Antoni et al. 2017). However, the prediction of treatment response and disease course still remains a challenging task, due to the cellular and molecular heterogeneity of bladder tumors as well as existing limitations on disease staging and grading (Babjuk et al. 2017; Kamat et al. 2016; Witjes et al. 2014). The lack of accurate and personalized disease prognosis, along with the high recurrence and progression rates of NMIBC, as well as the metastatic potential of MIBC, drives the lifelong surveillance of the patients and the increased healthcare system costs (Svatek et al. 2014). Consequently, the identification of novel tumor markers, able to support personalized prognosis, will improve disease management, increase the effectiveness of healthcare system, and benefit patients' quality-of-life.

The *TP63* gene (3q27–29), also known as *p63*, was independently cloned by multiple groups (Osada et al. 1998; Schmale and Bamberger 1997; Senoo et al. 1998; Yang et al. 1998) and determined to be a member of the p53 gene family of transcription factors (p53/p63/p73) (Kaelin 1999). *TP63* expression is regulated by two distinct promoters, leading to two different polypeptide products, those containing the transactivation TA domain at the N-terminus, known as TAp63, and those lacking the TA domain, referred as ΔNp63. In addition, C-terminal alternative splicing events of the primary transcripts result in five different isoforms (α, β, γ, δ, and ε) of either TAp63 or ΔNp63 products (Mangiulli et al. 2009; Yang and McKeon 2000).

Based on their structural similarity, it was believed that p63 functions would be similar to p53, regarding cell cycle control and tumor-suppressive role. However, even though TAp63 isoforms might behave like p53, as they are capable of transactivating p53 downstream targets, ΔNp63 isoforms display an antagonistic behavior, acting as a dominant-negative transcriptional repressor, opposing to the transactivation activity of the p53 family (Bergholz and Xiao 2012). The role of p63 in tumorigenesis and cancer progression is controversial, as both tumor-suppressive and oncogenic properties have been demonstrated (Flores 2007). In general, TAp63 acts as a tumor suppressor (Malaguarnera et al. 2005), while ΔNp63 displays the highest expression levels in basal epithelial cells and exhibits oncogenic features promoting epithelial tumorigenesis (Hibi et al. 2000; Kumakura et al. 2017).

Concerning BlCa, there is compelling evidence that p63 can act as a protective marker (Chen et al. 2018; Gaya et al. 2015). In this regard, ΔNp63 inhibits cancer progression and epithelial-to-mesenchymal transition (EMT) via regulating genes involved in cell adhesion, motility, and migration, including N-cadherin, b-catenin, and ZEB1/ZEB2 (Fukushima et al. 2009; Koga et al. 2003a; Tran et al. 2013). Moreover, reduced ΔNp63 protein levels have been associated with worse prognosis of BlCa patients (Urist et al. 2002). However, a complete evaluation of the clinical significance of *TP63* transcription in disease prognosis has not been carried out yet. Herein, we have performed the expression analysis of ΔNp63 and TAp63 transcripts in bladder tumors and normal bladder urothelium, and we have evaluated their potential clinical use in improving BlCa prognosis as well as achieving a more accurate risk stratification of the patients.

Patients and methods

Screening cohort

In the present study, 342 fresh-frozen bladder tissue specimens of our screening cohort were analyzed. Bladder tumors were obtained from 182 patients that underwent transurethral resection of bladder tumors (TURBT) for primary NMIBC or radical cystectomy (RC) for primary MIBC at the 'Laiko' General Hospital, Athens, Greece. From 121 patients of the screening cohort, adjacent normal bladder tissues were available, following the pathologist's assessment for the absence of dysplasia or carcinoma in situ (CIS). Moreover, 39 healthy bladder tissue specimens were collected from benign prostate hyperplasia patients.

The European Organization for Research and Treatment of Cancer (EORTC) guidelines were used for the risk-group stratification of the NMIBC patients. None of the patients received any kind of neoadjuvant treatment prior to surgical resection. Adjuvant treatment was administered to NMIBC patients according to European Association of Urology (EAU) guidelines, while MIBC patients did not receive any form of adjuvant therapy. NMIBC monitoring included cystoscopy and urinary cytology according to EAU guidelines. MIBC patients were followed up by renal ultrasound at 3 months and thoracoabdominal CT/MRI every 6 months. Additional renal ultrasound and thoracoabdominal CT/MRI, as well as bone scan or brain MRI were carried out following symptoms.

The study was approved by the ethical board of "Laiko" General Hospital and performed in accordance with the ethical standards of the 1975 Declaration of Helsinki, as revised in 2008. Informed consent was provided by all the participated patients.

Validation cohorts

The Hedegaard et al. (2016) ($n=476$) (Hedegaard et al. 2016) and TCGA (The Cancer Genome Atlas, provisional) ($n=413$) (Robertson et al. 2017) cohorts were used as validation cohorts for NMIBC and MIBC, respectively. Hedegaard et al. (2016)'s cohort ($n=476$) consisted of 460 NMIBC (Ta: 345, T1: 112, CIS: 3) and 16 MIBC patients, analyzed by paired-end whole transcriptome, strand-specific RNA-seq (Illumina HiSeq platform). TCGA (provisional) cohort ($n=413$) included 409 MIBC patients, and mRNA expression profiles generated by paired-end whole transcriptome RNA-seq (Illumina HiSeq platform). The clinical and normalized expression publicly available data were downloaded for Hedegaard et al. 2016 cohort by EMBL-EBI ArrayExpress (accession number ArrayExpress: E-MTAB-4321; https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-4321/) and for TCGA cohort (provisional) by cbioportal (http://www.cbioportal.org/).

Extraction of total RNA

Total RNA was extracted following pulverization of 40–100 mg fresh-frozen tissue specimen, using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA concentration and purity were determined at 260 and 280 nm using BioSpec-nano UV–Vis Spectrophotometer (Shimadzu Corp., Kyoto, Japan), while RNA integrity was evaluated by agarose gel electrophoresis.

First-strand cDNA synthesis

Reverse transcription took place in a 20 µL reaction containing 1 µg of total RNA template, 10 mM dNTP Mix, 50U of M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 40U RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), and 100 µM oligo(dT) primer. Reverse transcription was performed at 37 °C for 60 min, while the enzyme was inactivated at 70 °C for 15 min.

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) assays were developed and applied, using SYBR Green I dye, for the quantification of TAp63 and ΔNp63 transcript levels. Based on published sequences (NCBI RefSeq: NM_001114980.1 for ΔNp63, NM_003722.5 for TAp63 and NM_000194.3 for *HPRT1*) and in silico analysis, specific primers for ΔNp63 (F:5′-GGAAAACAATGCCCAGACTC-3′, R:5′-GAAGGACACGTCGAAACTGTG-3′), TAp63 (F:5′-GGTGCGACAAACAAGATTGAG-3′, R:5′-GAAGGACACGTCGAAACTGTG-3′), and *HPRT1* (F:5′-TGGAAAGGGTGTTTATTCCTCAT-3′, R:5′-ATGTAATCCAGCAGGTCAGCAA-3′) were designed and used to generate a 250 bp ΔNp63-, a 294 bp TAp63-, and a 151 bp *HPRT1*-specific amplicon. The 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) was used for the qPCR reactions. The 10 µL reaction mixture included 5 µL Kapa SYBR Fast Universal 2X qPCR Master Mix (Kapa Biosystems), 10 ng cDNA template and 100 nM of each specific primer for *HPRT1* and TAp63, or 75 nM of ΔNp63 specific primers. The thermal protocol consisted of 95 °C for 3 min for polymerase activation, and 40 cycles of denaturation at 95 °C for 15 s and primer annealing and extension at 60 °C for 1 min. Following amplification reactions, dissociation curves and agarose gel electrophoresis were performed for the discrimination of specific amplicons from non-specific products or primer dimers.

The reactions were performed in duplicate for each tested sample and target, and the average CT was calculated for quantification, according to the $2_{T}^{-\Delta\Delta C}$ relative quantification (RQ) method. *HPRT1* was used as an endogenous reference gene for normalization and the RT112 bladder cancer cell line as a calibrator.

Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics 20 software (IBM Corp., Armonk, New York, USA). The non-parametric Mann–Whitney *U* test and the Wilcoxon Singed-Rank test were applied to analyze transcript levels differences between bladder tumors and healthy bladder urothelium or normal adjacent bladder tissues, respectively. The Mann–Whitney *U* test was used to correlate transcripts levels with muscle-invasive disease and tumor grade, while the non-parametric Kruskal–Wallis test was performed to analyze the association of transcripts levels with tumor stage and EORTC-risk group. The value of ΔNp63 transcript to discriminate bladder tumors from normal bladder specimens was assessed by ROC curve and logistic regression analysis.

Survival analysis was performed by Kaplan–Meier curves using log-rank test and Cox proportional regression analysis, evaluating disease recurrence and progression for NMIBC or patient's death for MIBC as clinical endpoint events. The X-tile algorithm (Camp et al. 2004) was used for the determination of optimal cut-off values of ΔNp63 transcript levels in NMIBC and MIBC patient groups in the screening cohort, while the median *TP63* expression was used for the survival analysis of the Hedegaard et al. and TCGA validation cohorts. Bootstrap Cox proportional regression analysis based on 1000 bootstrap samples was performed for internal validation. The clinical net benefit of the multivariate prognosis prediction models on patients' survival outcome was evaluated by decision curve analysis according to Vickers et al. (Vickers and Elkin 2006) using the STATA 13 software (StataCorp LLC, College Station, TX, USA).

Results

Baseline clinical and experimental data

The screening cohort consisted mainly of males (84.6%) with a median age of 70 years. Concerning disease features, 66.5% and 33.5% of the patients were diagnosed and treated for primary NMIBC (TaT1) or MIBC (T2–T4), respectively. According to WHO 2004 grading system, 42.3% of the tumors were stratified as low grade (LG), while the remaining 57.7% as high grade (HG). Within the T1 cohort, 67.2% of the tumors were HG, while most of MIBC patients (96.5%) displayed HG tumors. Regarding the EORTC-risk stratification of NMIBC, 13.2%, 32.2%, and 54.5% of the patients were classified as low, intermediate, and high risk, respectively.

Concerning patients' monitoring, 157 patients were successfully followed up and included in survival analysis, whereas 25 patients were excluded due to unclear or insufficient monitoring data. During a median follow-up time (reverse Kaplan–Meier method) of 31 months (95% CI 28.50–33.50), of the 103 followed-up TaT1 patients, disease recurrence and progression were detected in 41 (39.8%) and 16 (15.5%) patients, respectively. The mean disease-free survival (DFS) and progression-free survival (PFS) of the NMIBC patients were 30.20 (95% CI 26.61–33.78) and 42.74 months (95% CI 39.90–45.58), respectively. Focusing on MIBC, 26 of the 54 follow-up patients died (48.1%), displaying mean overall survival (OS) of 28.99 months (95% CI 24.02–33.97).

The REMARK diagram of the study is included in Fig. 1, and patients' clinicopathological features are presented in detail in Table 1.

ΔNp63 are the predominant *TP63* transcripts expressed in bladder tissues and strongly upregulated in bladder tumors

The expression analysis highlighted that ΔNp63 are the main transcripts of *TP63* gene expression in bladder urothelium, whereas TAp63 transcripts are only minimally expressed and hardly detectable. According to the descriptive statistics of *TP63* expression in our screening cohort (Supplementary Table 1), TAp63 transcripts expression was not detected in approximately 53% and 81% of the analyzed bladder tumors ($n = 30$) and adjacent normal tissue specimens ($n = 26$), respectively. Due to the extremely low and in most cases

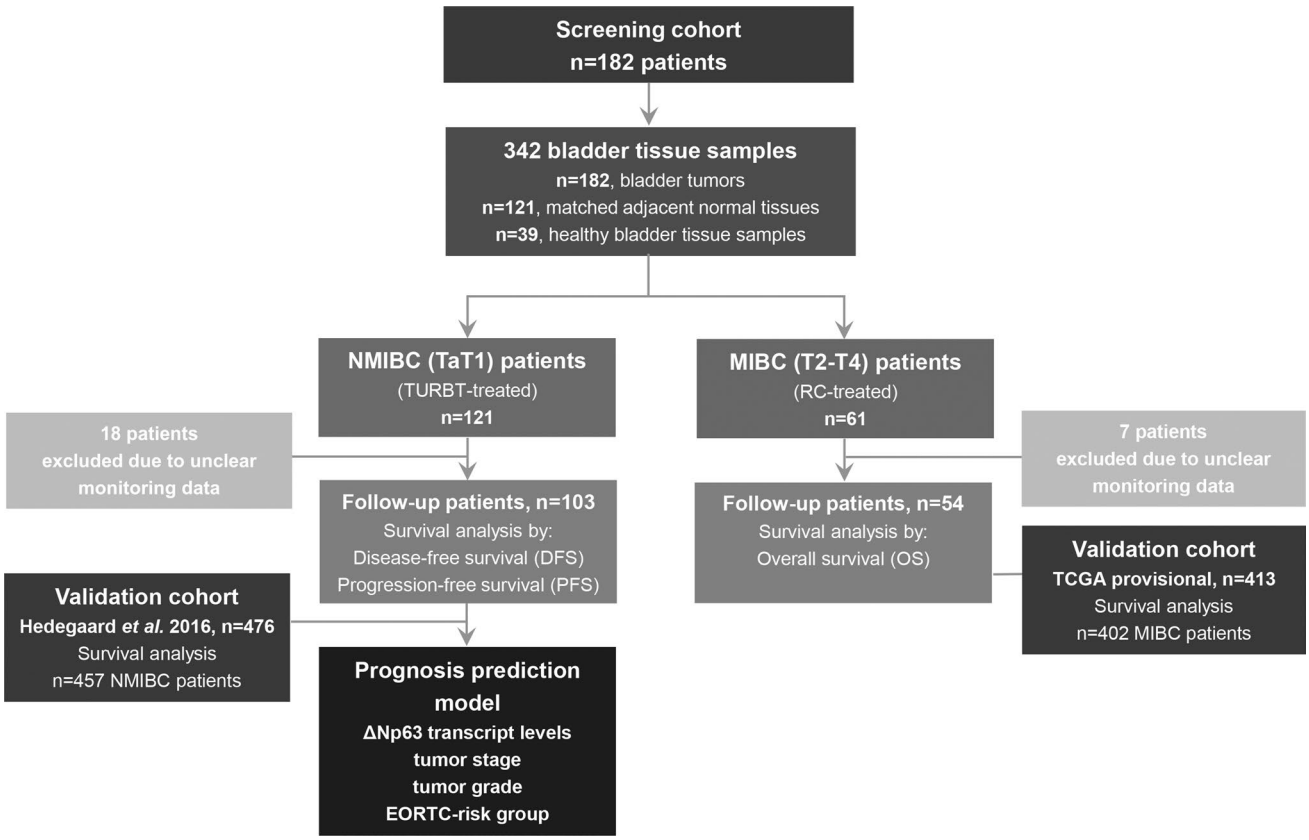


Fig. 1 REMARK diagram of the study

Table 1 Clinicopathological features of the screening BlCa cohort

Variable	No. of patients $n = 182$
Disease	
NMIBC (Ta, T1)	121 (66.5%)
MIBC (T2–T4)	61 (33.5%)
Tumor stage	
pTa	63 (34.6%)
pT1	58 (31.9%)
pT2	30 (16.5%)
pT3	15 (8.2%)
pT4	16 (8.8%)
Grade (WHO 2004)	
Low	77 (42.3%)
High	105 (57.7%)
Grade (WHO 1973)	
1	17 (9.3%)
2	68 (37.4%)
3	97 (53.3%)
Gender	
Male	154 (84.6%)
Female	28 (15.4%)
Non-muscle-invasive bladder cancer (NMIBC; TaT1)	
EORTC-risk group	
Low risk	16 (13.2%)
Intermediate risk	39 (32.2%)
High risk	66 (54.5%)
Disease monitoring	
Follow-up patients	103
Recurrence/progression	41 (39.8%)/16 (15.5%)
Event-free survival	62 (60.2%)/87 (84.5%)
Excluded from follow-up	18
Muscle-invasive bladder cancer (MIBC; T2–T4)	
Disease monitoring	
Follow-up patients	54
Alive	28 (51.9%)
Death	26 (48.1%)
Excluded from follow-up	7

undetected expression levels of the TAp63 transcript in bladder specimens, we decided to abandon the evaluation of its prognostic significance in the whole cohort of the study.

ΔNp63 transcripts levels were strongly upregulated in bladder tumors compared to healthy urothelium ($p < 0.001$) or matched adjacent normal specimens ($p < 0.001$) (Fig. 2). In particular, higher ΔNp63 transcript levels were detected in bladder tumors compared to the matched adjacent normal specimens in 62.8% of the enrolled patients (Fig. 2a). The elevated expression of ΔNp63 transcripts in bladder tumors was confirmed in NMIBC, where increased ΔNp63 expression in TaT1 tumors compared to matched normal specimens was observed in approximately 68.4% of the patients (Fig. 2c). Concerning MIBC, the slight increase of ΔNp63 transcript levels in muscle-invasive tumors (T2–T4) related to matched normal specimens (52.4%) was not statistically significant ($p = 0.199$; Fig. 2e).

The clinical significance of ΔNp63 upregulation for the discrimination of bladder tumors was evaluated by ROC curve (Fig. 2) and logistic regression analysis (Supplementary Table 2). Indeed, ΔNp63 could distinguish bladder tumors from control healthy specimens with (AUC: 0.885; 95% CI 0.837–0.933, $p < 0.001$), as well as from their matched adjacent normal counterparts (AUC: 0.640; 95% CI 0.578–0.702, $p < 0.001$). This discriminatory significance of ΔNp63 was also confirmed for TaT1 tumors compared to healthy controls (AUC: 0.934, 95% CI 0.891–0.976, $p < 0.001$) or matched normal specimens (AUC: 0.686, 95% CI 0.611–0.761, $p < 0.001$), as well as for muscle-invasive tumors related to healthy urothelium (AUC: 0.789, 95% CI 0.700–0.878, $p < 0.001$).

Downregulated ΔNp63 transcript levels are correlated with unfavorable prognostic features of BlCa

Despite the significantly elevated expression of ΔNp63 transcript in BlCa, its expression analysis related to BlCa clinical features revealed the significant association of the reduced ΔNp63 transcript levels with bladder tumors of aggressive clinical phenotype (Fig. 3). Significantly decreased ΔNp63 transcript levels were detected in muscle-invasive disease ($p < 0.001$), as well as in higher stage ($p < 0.001$) and grade ($p < 0.001$) tumors in our screening cohort (Fig. 3a). Focusing on TaT1 patients, downregulated ΔNp63 levels were highlighted in T1HG tumors compared to Ta and T1LG tumors ($p = 0.005$), as well as in high- and intermediate-EORTC-risk patients related to low-risk group ($p = 0.033$) (Fig. 3b). Supporting our results, the analysis of the validation cohorts clearly confirmed the downregulation of *TP63* expression in tumors of higher stage (Hedegaard et al., $p = 0.008$; Fig. 3C and TCGA, $p = 0.016$; Fig. 3d) and higher grade (TCGA, $p < 0.001$; Fig. 3d).

Loss of ΔNp63 transcript is correlated to significantly higher risk for recurrence and progression of NMIBC (TaT1) patients

Disease relapse and progression for NMIBC or patients' death for MIBC were used as clinical endpoint events for patients' survival analysis. Using the X-tile algorithm, the 45th and the 50th (median) percentile of the ΔNp63 transcript levels were adopted as optimal cut-off values for the survival analysis of NMIBC and MIBC patients, respectively. Concerning NMIBC, Kaplan–Meier curves highlighted the shorter DFS ($p = 0.013$; Fig. 3e) and PFS ($p = 0.008$;

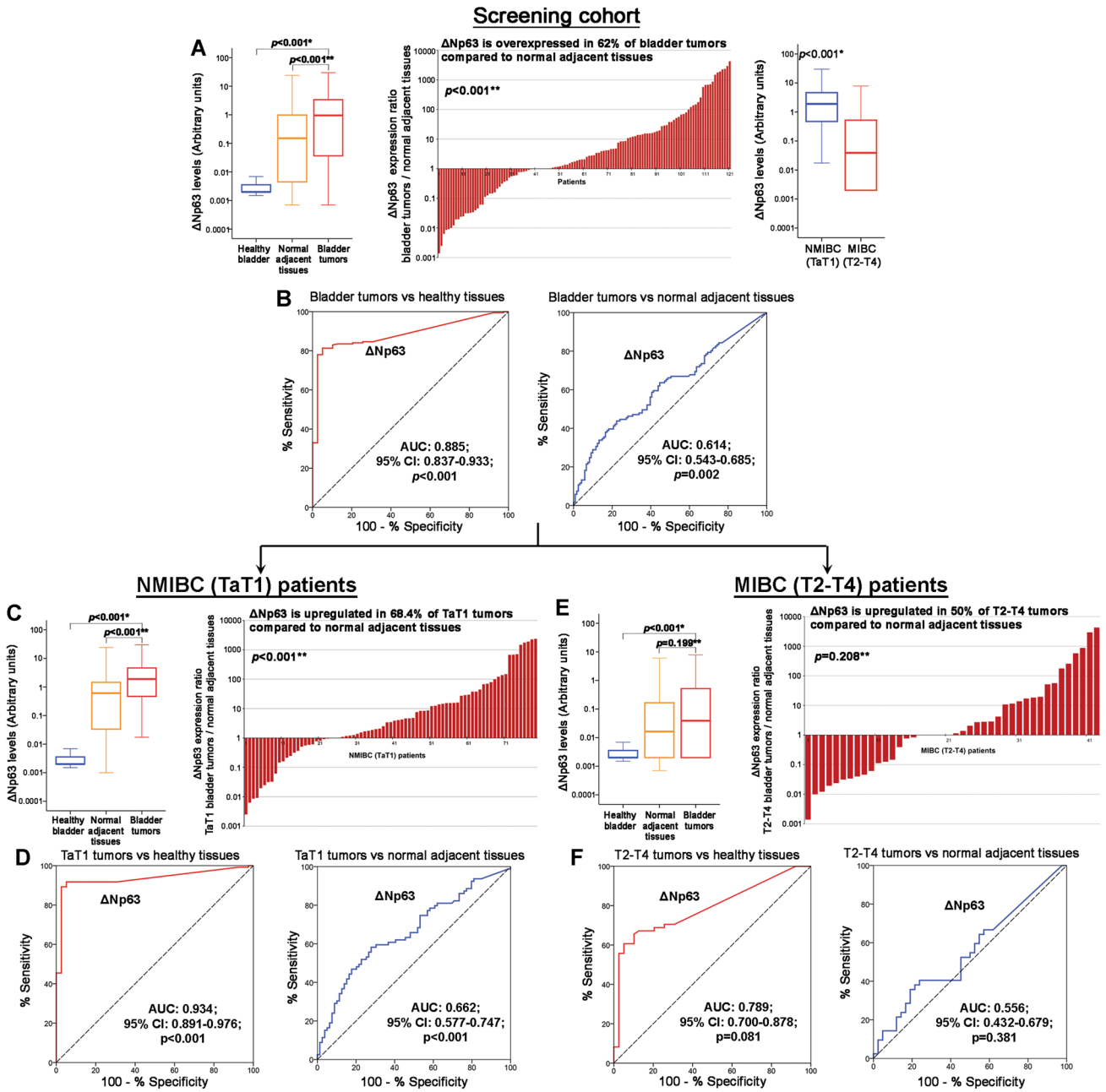


Fig. 2 Expression analysis of ΔNp63 transcripts in the screening cohort of bladder urothelial carcinoma patients. **a** Box plot of ΔNp63 transcripts levels in bladder tumors, matched normal adjacent tissues, and healthy tissues (left), bar graph of the ΔNp63 transcripts levels ratio in bladder tumors *vs* matched adjacent normal tissues of BlCa patients (middle), and box plot of ΔNp63 transcripts tumor levels in NMIBC and MIBC patients (right). *p* values calculated by Mann–Whitney *U* test (*) and Wilcoxon Signed-Rank test (**). **b** ROC curve analysis of ΔNp63 transcript expression for the discrimination of bladder tumors from healthy control specimens (left) or from matched normal adjacent tissues (right). *p* value calculated by Hanley and McNeil method. **c**, **e** Box plot of ΔNp63 transcripts levels in bladder tumors, matched normal adjacent tissues and healthy tissues (left), and bar graph of the ΔNp63 transcripts levels ratio in bladder tumors *vs* matched adjacent normal tissues (right) of **c** NMIBC (TaT1) patients and **e** MIBC (T2–T4) patients. *p* values calculated by Mann–Whitney *U* test (*) and Wilcoxon Signed-Rank test (**). **d**, **f** ROC curve analysis of ΔNp63 transcripts expression for the discrimination of **d** NMIBC (TaT1) patients from healthy controls (left) and of non-muscle-invasive tumors (TaT1) from matched normal adjacent specimens (right), **f** MIBC (T2–T4) patients from healthy controls (left) and of muscle-invasive tumors (T2–T4) from matched normal adjacent specimens (right). *p* value calculated by Hanley and McNeil method. AUC: area under the curve, 95% CI 95% confidence interval

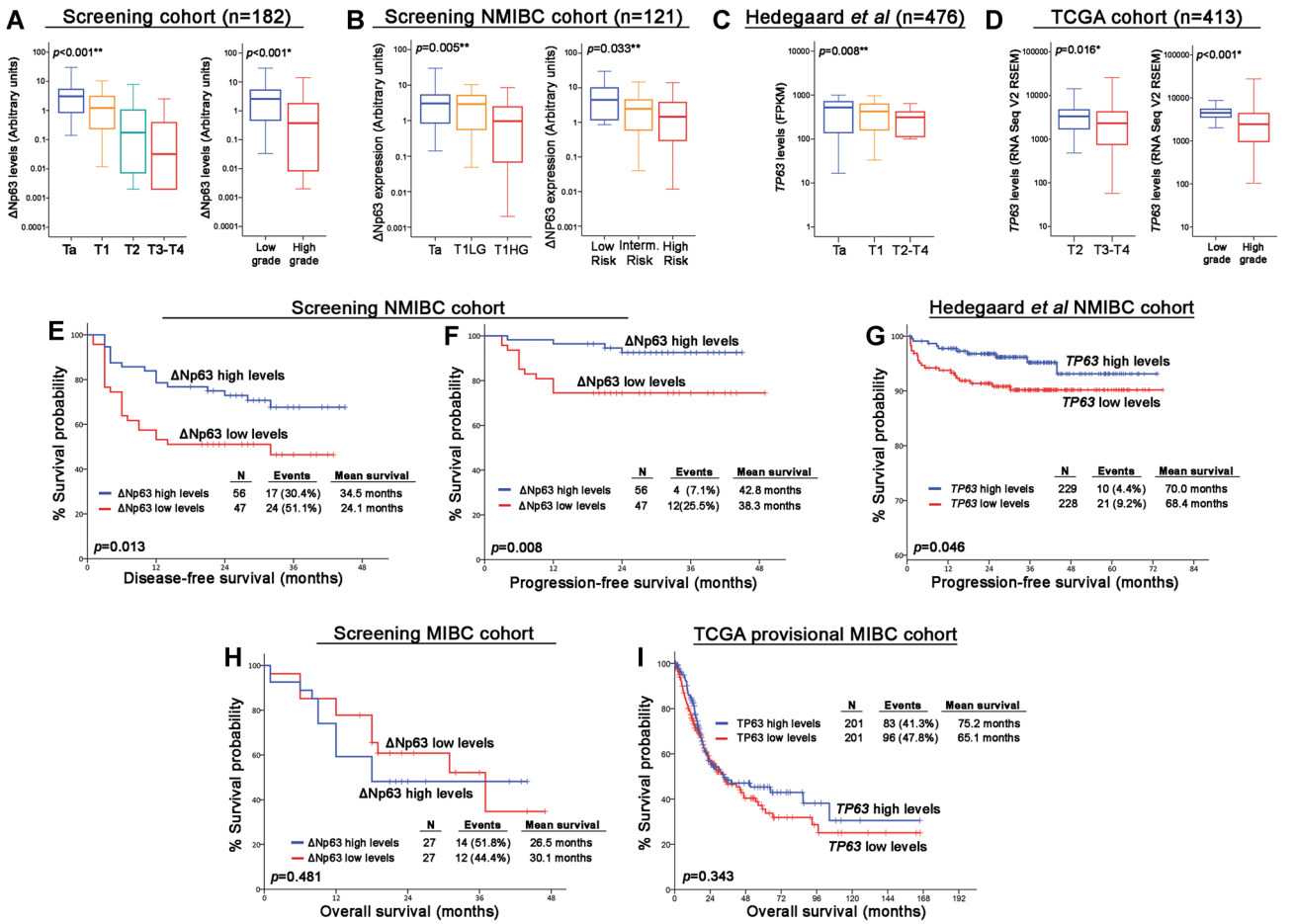


Fig. 3 Downregulated ΔNp63 transcript expression is strongly associated with unfavorable disease prognosis. **a**–**d** Box plots representing the correlation of ΔNp63 transcripts levels with **a** tumor stage (left) and tumor grade (right) in the screening BlCa cohort, **b** tumor staging/grading (left) and EORTC-risk group (right) in the screening NMIBC cohort, **c** tumor stage in the Hedegaard et al. (2016) validation cohort, **d** tumor stage (left) and tumor grade (right) in the TCGA provisional validation cohort. *p* values calculated by Mann–Whitney *U* test (*) and Kruskal–Wallis test (**). **e**–**i** Kaplan–Meier survival curves for the **e** disease-free survival (DFS) and **f** progression-free survival (PFS) of the screening NMIBC (TaT1) cohort, **g** PFS of the Hedegaard et al. (2016) NMIBC validation cohort, **h** overall survival (OS) of the screening MIBC (T2–T4) cohort, and **i** OS of the TCGA provisional MIBC validation cohort, according to ΔNp63 transcripts levels. *p* values of the Kaplan–Meier survival analysis calculated by log-rank test

Fig. 3f) expectancy of the TaT1 patients with lower ΔNp63 expression compared to patients over-expressing ΔNp63 transcripts. In this regard, univariate Cox regression analysis (Fig. 4, Supplementary Table 3) confirmed the significantly stronger risk for disease recurrence (HR: 2.120; 95% CI 1.136–3.955; $p = 0.018$) and progression to invasive disease stages (HR: 4.097; 95% CI 1.319–12.723; $p = 0.015$) of the NMIBC patients with reduced ΔNp63 expression. Moreover, multivariate Cox models (Fig. 4, Supplementary Table 3), adjusted for ΔNp63 transcript levels, tumor stage, grade, EORTC-risk group, patients' gender and age, strongly verified ΔNp63 transcript loss as an independent molecular marker for the prediction of NMIBC patients' relapse (HR: 2.730; 95% CI 1.312–5.681; $p = 0.007$) and progression (HR: 7.757; 95% CI 1.463–41.139; $p = 0.016$) following treatment. Internal validation using bootstrap Cox regression models confirmed the independent prognostic significance of ΔNp63 downregulation for NMIBC outcome. Unfortunately, the survival analysis of MIBC patients did not highlight any statistically significant correlation of ΔNp63 levels with patients' OS (Fig. 3h).

The analysis of the Hedegaard et al.'s validation cohort strongly confirmed our findings regarding the adverse treatment outcome of the NMIBC patients under-expressing *TP63* (Fig. 3). More precisely, Hedegaard et al.'s cohort demonstrated significantly higher risk for disease progression to muscle-invasive stages following treatment and the shorter PFS expectancy of the NMIBC patients (TaT1) with reduced *TP63* expression ($p = 0.046$; Fig. 3g). Finally, the survival analysis of the TCGA cohort did not highlight any

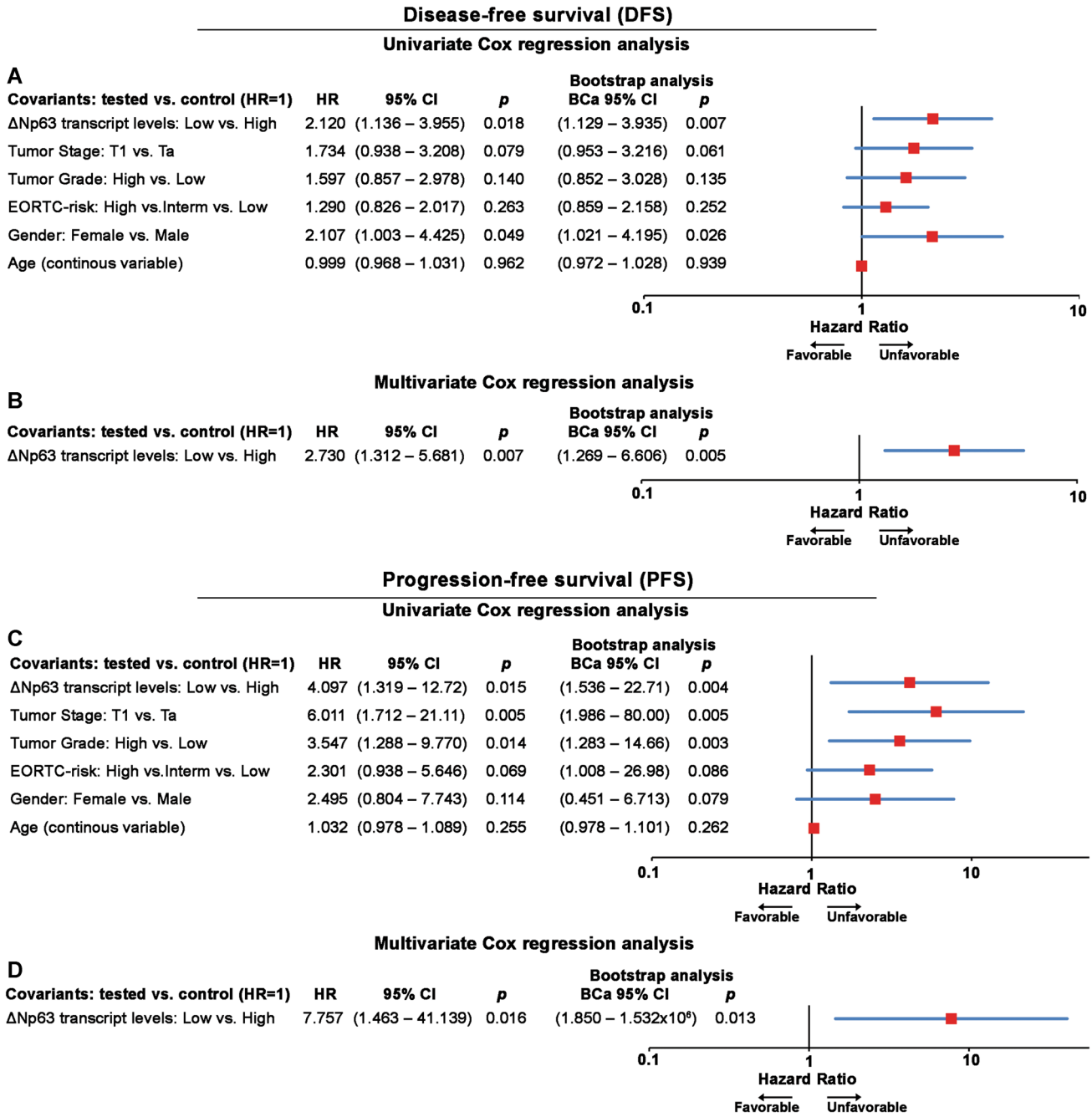


Fig. 4 Loss of ΔNp63 expression represents an independent predictor of NMIBC patients' higher risk for early relapse and progression to invasive disease. Forest plots of the univariate (**a**, **c**) and multivariate (**b**, **d**) Cox regression analysis for the disease-free survival (DFS) and progression-free survival (PFS) of the NMIBC (TaT1) patients of the screening cohort. Multivariate analysis adjusted for ΔNp63 transcripts levels, tumor stage, tumor grade, EORTC-risk group, and patient's gender and age. Internal validation was performed by bootstrap Cox proportional regression analysis based on 1000 bootstrap samples. HR: Hazard Ratio, 95% CI 95% confidence interval of the estimated HR, BCa 95% CI bootstrap bias-corrected and accelerated 95% confidence interval of the estimated HR based on 1000 bootstrap samples

statistically significant correlation of ΔNp63 levels with MIBC patients' survival outcome (Fig. 3i).

The evaluation of ΔNp63 transcript levels enhances the prognostic value of the clinically established markers for NMIBC

The strong independent prognostic utility of ΔNp63 transcripts for NMIBC patients motivated us to study its ability to strengthen the clinical value of the conventionally used disease prognostic markers. More precisely, we have evaluated the integration of ΔNp63 with tumor staging and grading, as well as EORTC-risk group, as they constitute the established and clinically used prognostic markers for NMIBC, in improving prediction of disease post-treatment outcome.

The combined evaluation of ΔNp63 expression with the above-mentioned clinical markers resulted in superior positive prediction of patients' relapse and progression (Fig. 5). Indeed, loss of ΔNp63 levels could effectively stratify patients according to their risk for early relapse within T1HG and Ta/T1LG patient groups ($p = 0.025$; Fig. 5a), as well as to distinguish T1HG patients at increased risk for disease progression to invasive disease stages ($p < 0.001$; Fig. 5b). Similarly, evaluation of ΔNp63 expression could benefit patients' stratification according to EORTC guidelines. Indeed, intermediate/high-risk patients over-expressing ΔNp63 transcripts displayed superior post-treatment outcome, and analogous to low-risk group, compared to intermediate/high-risk patients with loss of ΔNp63 expression suffering from increased risk for early relapse ($p = 0.028$; Fig. 5c) and progression ($p < 0.001$; Fig. 5d). In this regard, decision curve analysis according to Vickers et al. confirmed the superior clinical net benefit of the multivariate models combining ΔNp63 loss with the established clinical markers of NMIBC for the prognosis of both patients' relapse (Fig. 5e) and progression (Fig. 5f), compared to the model of the clinical prognostic markers alone.

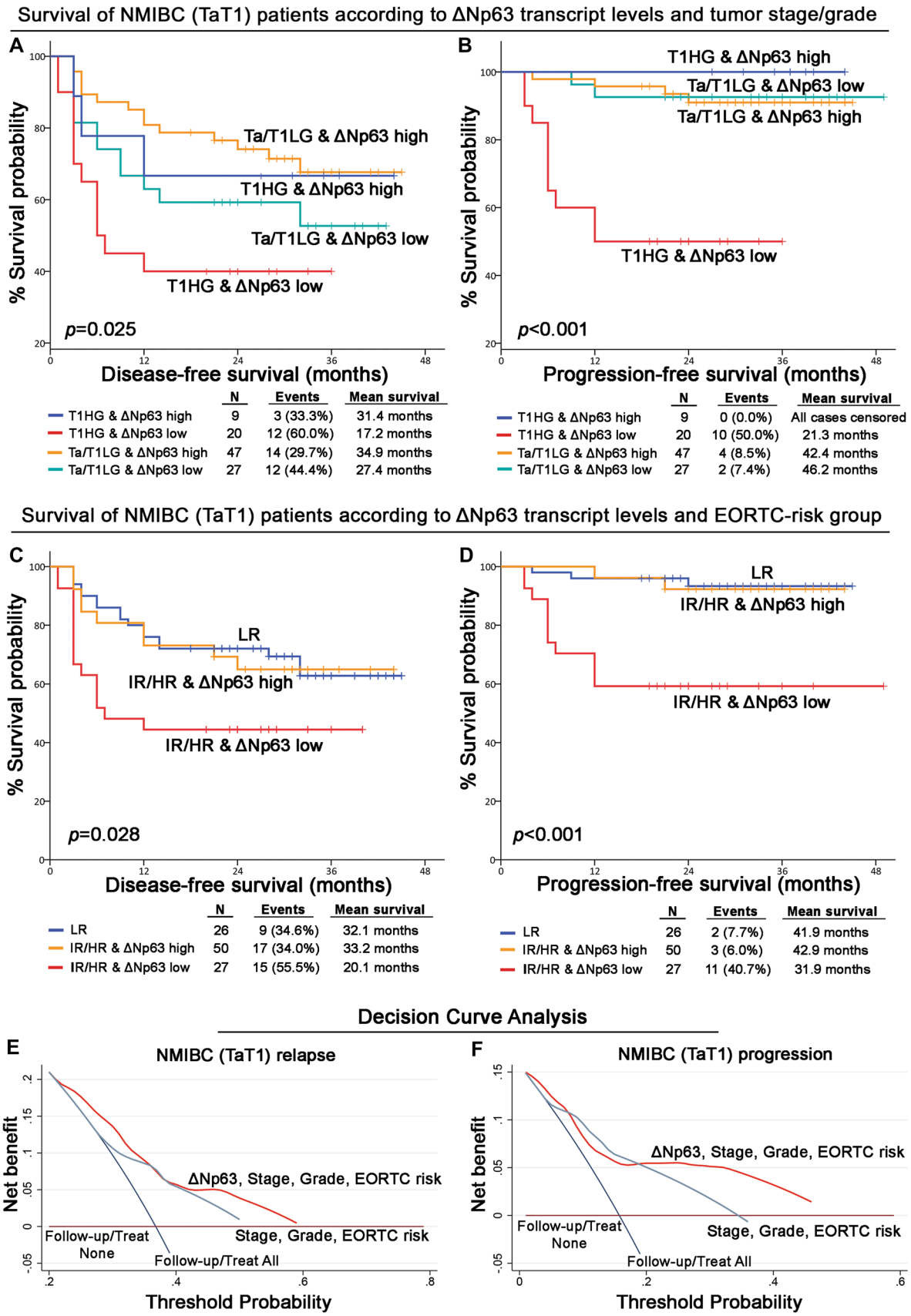
Discussion

Urothelial carcinoma of the bladder is the most common malignancy affecting the male urinary tract, and is considered as a highly heterogeneous malignancy both in molecular level and treatment outcome (Prasad et al. 2011). Despite the recent technological advances in disease diagnosis and therapy, current prognostic tools cannot ensure optimum disease management. The high morbidity and mortality rates as well as the lack of precision prognostics are the underlying limitations for the non-personalized disease management and the required lifelong surveillance of patients (Donovan and Cordon-Cardo 2014). Over the past several years, considerable progress has been made in the elucidation of the genetic/molecular driver events in bladder tumorigenesis (Castillo-Martin et al. 2010; Knowles and Hurst 2015; Rampias et al. 2014; Van Batavia et al. 2014), and consequently, the identification of novel molecular markers (Avgeris et al. 2015; Avgeris et al. 2018; Tsikrika et al. 2018) and therapeutic targets (Felsenstein and Theodorescu 2018), towards improving disease prognosis and limiting unnecessary interventions and healthcare system costs (Jordan and Meeks 2018). The aim of the present study was to evaluate the clinical significance of ΔNp63 and TAp63 transcript variants of *TP63* expression, in improving disease prognosis and prediction of patients' treatment outcome.

The analysis of our screening cohort highlighted that ΔNp63 represents the main transcript of *TP63* expression in bladder urothelium, while TAp63 transcripts are only minimally expressed (Choi et al. 2012; Comperat et al. 2007; Koga et al. 2003a). In this regard, ΔNp63 expression was significantly upregulated in bladder tumors compared to normal urothelium, while ROC curve and logistic regression analysis demonstrated the ability of ΔNp63 transcript levels to distinguish bladder tumors from normal urothelium. However, the loss of ΔNp63 transcript levels was associated with unfavorable clinical prognostic markers. More precisely, downregulated ΔNp63 levels were observed in muscle-invasive (T2–T4) compared to superficial (TaT1) tumors and in HG tumors. Regarding NMIBC clinical features, under-expression of ΔNp63 transcripts was detected in T1HG tumors and high-EORTC-risk TaT1 patients.

The survival analysis confirmed the significant unfavorable prognostic value of ΔNp63 expression loss for NMIBC patients' outcome. Indeed, Kaplan–Meier curves and univariate Cox regression analysis highlighted the higher risk for post-treatment recurrence and progression of the TaT1 patients with reduced ΔNp63 transcript levels compared to patients over-expressing ΔNp63 transcripts. Moreover, multivariate Cox models demonstrated the adverse post-treatment outcome of the NMIBC patients with ΔNp63 loss independently of the disease clinical markers tumor stage, grade and EORTC-risk stratification, as well as patients' age and gender.

TP63 expression data from Hedegaard et al. and TCGA provisional cohorts were analyzed to validate our findings, as TAp63 transcript is poorly expressed in bladder urothelium. The study of the validation cohorts clearly confirmed the unfavorable nature of *TP63* downregulation in BlCa prognosis. More precisely, the reduced *TP63* transcription was strongly correlated with muscle-invasive disease and higher tumor staging and grading. In addition, Hedegaard et al.'s validation cohort for NMIBC strongly confirmed the higher risk for post-treatment progression to muscle-invasive disease of the NMIBC patients under-expressing *TP63*. On the other hand and similar to the survival analysis of the


Survival of NMIBC (TaT1) patients according to ΔNp63 transcript levels and tumor stage/grade
A
% Survival probability
Ta/T1LG & ΔNp63 high
T1HG & ΔNp63 high
Ta/T1LG & ΔNp63 low
T1HG & ΔNp63 low
p=0.025
Disease-free survival (months)
N Events Mean survival
T1HG & ΔNp63 high 9 3 (33.3%) 31.4 months
T1HG & ΔNp63 low 20 12 (60.0%) 17.2 months
Ta/T1LG & ΔNp63 high 47 14 (29.7%) 34.9 months
Ta/T1LG & ΔNp63 low 27 12 (44.4%) 27.4 months
B
% Survival probability
T1HG & ΔNp63 high
Ta/T1LG & ΔNp63 low
Ta/T1LG & ΔNp63 high
T1HG & ΔNp63 low
p<0.001
Progression-free survival (months)
N Events Mean survival
T1HG & ΔNp63 high 9 0 (0.0%) All cases censored
T1HG & ΔNp63 low 20 10 (50.0%) 21.3 months
Ta/T1LG & ΔNp63 high 47 4 (8.5%) 42.4 months
Ta/T1LG & ΔNp63 low 27 2 (7.4%) 46.2 months
Survival of NMIBC (TaT1) patients according to ΔNp63 transcript levels and EORTC-risk group
C
% Survival probability
LR
IR/HR & ΔNp63 high
IR/HR & ΔNp63 low
p=0.028
Disease-free survival (months)
N Events Mean survival
LR 26 9 (34.6%) 32.1 months
IR/HR & ΔNp63 high 50 17 (34.0%) 33.2 months
IR/HR & ΔNp63 low 27 15 (55.5%) 20.1 months
D
% Survival probability
LR
IR/HR & ΔNp63 high
IR/HR & ΔNp63 low
p<0.001
Progression-free survival (months)
N Events Mean survival
LR 26 2 (7.7%) 41.9 months
IR/HR & ΔNp63 high 50 3 (6.0%) 42.9 months
IR/HR & ΔNp63 low 27 11 (40.7%) 31.9 months
Decision Curve Analysis
E
NMIBC (TaT1) relapse
Net benefit
ΔNp63, Stage, Grade, EORTC risk
Stage, Grade, EORTC risk
Follow-up/Treat None
Follow-up/Treat All
Threshold Probability
F
NMIBC (TaT1) progression
Net benefit
ΔNp63, Stage, Grade, EORTC risk
Stage, Grade, EORTC risk
Follow-up/Treat None
Follow-up/Treat All
Threshold Probability

◂**Fig. 5** Multivariate prognosis prediction models integrating ΔNp63 transcripts expression with the established clinically disease markers result in superior clinical benefit for the prediction of NMIBC (TaT1) relapse and progression. **a**–**d** Kaplan–Meier curves of the disease-free survival (DFS) and progression-free survival (PFS) of the NMIBC (TaT1) patients according to the integration of ΔNp63 transcription loss with (**a**, **b**) tumor stage and grade, and **c**, **d** EORTC-risk-group stratification. *p* values calculated by log-rank test. *T1HG* T1 high grade, *T1LG* T1 low grade, *LR* low risk, *IR* intermediate risk, *HR* high risk. **e**, **f** Decision curves of the multivariate prognosis prediction models for NMIBC (TaT1) relapse (**e**) and progression (**f**). Net benefit is plotted against various ranges of threshold probabilities

screening MIBC cohort, the analysis of the TCGA validation cohort for MIBC did not show any statistically significant correlation with MIBC patients' survival.

In general, p63 displays a critical role in the control of cell differentiation, proliferation, and survival. It has been documented that ΔNp63, the predominantly expressed *TP63* transcript in epithelial cells, is required for normal tissue development and plays a crucial role in cell cycle and cell growth regulation (Signoretti et al. 2000). Focusing on BlCa, our findings are in line with previous studies highlighting the contradictory role of ΔNp63 in tumor establishment and disease progression, demonstrating oncogenic and tumor-suppressive properties, respectively (Comperat et al. 2007; Gaya et al. 2015; Park et al. 2000).

The oncogenic role of ΔNp63 in bladder tumorigenesis relies on its ability to act as a dominant-negative factor of G1 cycle arrest, which is regularly induced by p53 and TAp63 (Park et al. 2000). Therefore, ΔNp63 over-expression has been suggested to accelerate papilloma formation and facilitate early stage bladder tumorigenesis, through the promotion of tumor cell proliferation and survival as well as the inhibition of senescence and apoptotic death (Comperat et al. 2007; Moses et al. 2019).

Focusing on disease progression, ΔNp63 acts protectively by possessing tumor-suppressive and anti-metastatic activities. In this regard, ΔNp63 has been documented to regulate the expression of cell adhesion, migration, and motility genes and thus to control EMT of bladder tumor cells (Chen et al. 2018). Loss of ΔNp63 transcript is associated with EMT-like changes and aggressive cancer cell phenotype (Barbieri et al. 2006), while the reduced ΔNp63 protein levels are significantly correlated with lower b-catenin and Uroplakin III expression, as well as higher ZEB1/ZEB2 levels, promoting tumor progression (Koga et al. 2003a; Koga et al. 2003b; Tran et al. 2013). Finally, p63 has been reported to be essential for maintaining FGFR3 expression and signaling in bladder cells (Sayan et al. 2010; Sjodahl et al. 2012), whereas the reduced ΔNp63 protein levels have been previously associated with patients' poor prognosis (Gaya et al. 2015; Urist et al. 2002).

Taking advantage of ΔNp63 independent clinical value for NMIBC, we have assessed ΔNp63 additional impact in improving the prognostic performance of the established and clinically used disease markers. Indeed, the integration of ΔNp63 transcripts loss resulted in improved specificity of NMIBC patients' risk stratification and superior positive prediction for disease relapse and progression within Ta/T1LG and T1HG cohorts, as well as within intermediate/high-EORTC-risk patients. Finally, decision curve analysis confirmed the superior clinical benefit of the prediction model incorporating ΔNp63 loss for NMIBC relapse and progression compared to the model of clinically used prognostic markers alone.

In conclusion, ΔNp63 is the main *TP63* transcript expressed in bladder urothelium and is significantly upregulated in bladder tumors compared to normal bladder tissues. Interestingly, ΔNp63 transcripts loss was associated with unfavorable disease features, such as invasive disease stages, HG tumors, and high-EORTC-risk group of the NMIBC patients. Regarding disease outcome, under-expression of ΔNp63 transcripts resulted in higher risk of early relapse and progression to invasive disease stages of NMIBC patients, independently of tumor stage, grade, EORTC-risk score, and patient's age and gender. Hedegaard et al. 2016 validation cohort clearly supported our findings regarding the unfavorable nature of ΔNp63 transcripts under-expression for NMIBC outcome. Moreover, the integration of ΔNp63 transcript loss with the clinically used disease prognostic markers resulted in improved risk stratification of NMIBC patients, superior specificity in predicting post-treatment relapse, and progression to invasive stages, and finally, decision curve analysis verified the higher clinical net benefit for NMIBC patients' management.

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Compliance with ethical statements

Conflict of interest The authors declare no conflict of interest.

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