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CLINICAL STUDY

Expression of BCL2, TP53, FOXA1, and GATA3 in pTa bladder cancer recurrence

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ABSTRACT

OBJECTIVES: In this study, we analyzed pTa bladder cancer (BC) for molecular markers BCL2, TP53, FOXA1, and GATA3 in relation to cancer recurrence.

METHODS: We analyzed samples of 79 patients with the pTa stage of BC using a real-time polymerase chain reaction (real-time PCR) between September 2018 and September 2020. The expression levels of BCL2, TP53, FOXA1, and GATA3 were compared with homologous non-tumor bladder tissue.

RESULTS: Expression of FOXA1, GATA3, and TP53 was significantly higher ($p<0.01$) in NMIBC samples compared to homologous non-tumor tissue. The expression of TP53 and FOXA1 in pTa was significantly lower ($p<0.01$) in the high-grade (HG) tumor when compared to the low-grade (LG) tumor. In contrast, the relative quantification (RQ) of GATA3 was significantly higher ($p<0.01$) in HG pTa. Patients with recurrence (pTa=33) had significantly higher expression of TP53, and GATA3 ($p<0.01$), and the gene of FOXA1 ($p<0.01$) had a significantly lower expression when compared to pTa tumors without recurrence. The expression of Bcl-2 was not statistically significant.

CONCLUSION: Our results, indicate, that comparing expression levels of these genes in cancer and cancer-free tissue could provide valuable data, as patients with pTa BC recurrence within up to 54 months of follow-up had a significantly higher RQ of TP53, GATA3, and FOXA1 when compared to pTa BC patients without recurrence *(Tab. 2, Fig. 8, Ref. 54)*. Text in PDF *www.elis.sk*

KEY WORDS: bladder cancer, gene expression, recurrence, GATA3, FOXA1, TP53, BCL2

Introduction

Bladder cancer (BC) is the fifth most common malignancy and the second most commonly diagnosed urogenital tumor after prostate cancer in Europe. Worldwide, BC is the 10th most common cancer diagnosed, with around 550,000 new cases annually (1). Its incidence is four times higher in men than in women, and independent from anticoagulant therapy (2, 3). Approximately 70% of BC is classified as non-muscle invasive bladder cancer (NMIBC) at the time of diagnosis (4, 5). NMIBC is a heterogenous disease that tends to recur and even progress to muscle-invasive bladder cancer (MIBC) (6). Even though NMIBC has a better prognosis when compared to MIBC in terms of cancer-specific survival, a significant group of patients with NMIBC will progress to MIBC with a poor prognosis (7). Regarding NMIBC recurrence, most NMIBC patients will recur over time (up to 80%) (6). The diagnosis and treatment of BC are based on cystoscopy and transurethral resection of the bladder tumor (TUR-BT). TUR-BT may eradicate NMIBC, but the frequent recurrence of these tumors requires regular follow-up and scheduled cystoscopies (8).

To predict the risks of NMIBC recurrence and progression, the EORTC Genito-Urinary Cancer Group (GUCG) published a scoring system and risk tables based on the World Health Organization (WHO) 1973 classification in 2006 (9). This system is based on clinical and pathological factors (number of tumors, tumor diameter, prior recurrence rate, T category, concurrent CIS, WHO 1973 tumor grade). In 2021, the European Association of Urology (EAU) introduced an updated scoring system and created risk groups based on clinical and pathological factors (tumor stage, WHO 1973 grade, WHO 2004/2016 grade, concomitant CIS, number of tumors, tumor size, and age) (10). However, this scoring model determines the risk of tumor progression, but not recurrence, as in the 2016 EORTC scoring model (11).

In the era of precision medicine, there is a search for markers that could provide more precise information about the risk of recur-

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rence and progression of NIMBC. Several promising molecular markers were studied, such as BCL2, TP53, FOXA1, and GATA3 often with contradictory results (11–15).

The Bcl protein family includes apoptosis-promoting (Bad, Bak, Bax, and Bid) and apoptosis-inhibiting Bcl-2 proteins (14). The balance between Bcl proteins decides whether apoptosis will be induced or whether the cells will continue to survive. Extracellular growth factors inactivate Bad, thereby suppressing Bcl-2 production and thus protecting against apoptosis (12). Increased expression of this gene leads to the blocking of apoptosis, causing continuous overexpression of BCL2 and BCLX anti-apoptotic genes which is linked to poor prognosis and chemotherapy resistance (13). Studies report BCL2 affecting tumorigenesis and tumor progression, facilitating either tumor growth or regression (15, 16).

TP53 is a transcription factor that has various functions, such as induction of apoptosis, inhibition of cell proliferation, arrest of the cell cycle, heat shock, hypoxia, and oncogene overexpression. Nuclear accumulation of p53 is a marker of poor prognosis in advanced BC (17, 18). The rise in TP53 expression is often caused by a mutant gene, that produces a stable mutant protein with higher chances of accumulation and loss of tumor suppressive function (19). The loss of function reduces the repressive function of p53 on the pro-proliferative FoxM1 and dysregulates the cell cycle in the G2/M-phase arrest (20).

Forkhead Box A1 (FOXA1) is expressed in normal cells and participates in normal genitourinary development. Reduced expression of the FOXA1 gene was correlated with increasing tumor stage. DeGraff and co-workers provided the first evidence linking the loss of FOXA1 expression with histological subtypes of BC (21).

Many studies confirm the association of these markers with prognosis, stage, and grade of BC (often with contradictory results). In contrast, there is a lack of studies focusing on their possible association with the recurrence of pTa BC (22–25).

GATA is a family of zinc finger transcription factors characterized by their ability to bind to the consensus DNA sequence (22, 26). GATA binding protein 3 (GATA3) contributes to hematopoiesis, particularly T-cell development, and differentiation, as well as the morphogenesis of other organs, such as the mammary gland and urogenital system (27, 28). GATA3 has been extensively studied in relation to breast morphogenesis and breast pathogenesis (29). GATA3 is known as a breast tumor suppressor as well as a urothelial marker, and its loss of expression is linked to progression and is often seen in HG-invasive BC (30). However, the GATA3 function in BC is still unknown (31).

In the present study, we investigated the expression levels of BCL2, TP53, FOXA1, and GATA3 by reverse transcription-quantitative polymerase chain reaction (RT-PCR) in pTa BC patients and their matched normal (non-tumor) bladder tissues in relation to BC prognosis and recurrence.

Materials and methods

We analyzed samples of 79 patients with the pTa stage of BC. Samples were collected during TUR-BT at the Department of Urology, Comenius University in Bratislava with a follow-up treatment according to the EAU guidelines. Each sample consisted of two parts, with one originating from the tumor and the other from non-tumor tissue. The second sample was subsequently divided: one part was stored at –80 °C as vital tissue, while the other underwent histopathological examination. After verifying and confirming the presence of tumor cells in the tumor sample at the absence the absence of tumor cells in the control tissue, vital tissue was processed for mRNA analysis. Staging and grading were done according to the eighth edition of the TNM staging system and to the WHO 2004/2016 classification, respectively (32). The sample collecting period was from September 2018 to September 2020, followed by 30 months of follow-up time until March 2023. Follow-up for individual subjects started on the day of sample collection during TUR-BT, which equals a follow-up period from 30 to 54 months. During this period, 33 patients had tumor recurrence, out of which 13 occurred in a close vicinity to the original tumor location. All patients who underwent immediate intravesical application of 50 mg epirubicin after TUR-BT were scheduled for follow-up cystoscopy according to EAU guidelines. All samples were collected in accordance with The Helsinki Declaration, after submitting informed consent. The study was approved by the Ethics Committee of the University Hospital in Bratislava under ref. number 05/2022. After excision, all samples were histopathologically examined by 2 independent experienced pathologists.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from sample sections of BC using GeneJET RNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. First-strand cDNA was synthesized from total RNA with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). The reaction was performed according to the protocol recommended by the manufacturer. The thermal cycling conditions were composed of 25 °C for 10 min, 50 °C for 15 min, and 85 °C for 5 min. The obtained cDNA was used as a template for quantitative PCR to determine the expression level of the selected genes. The samples of BC and their matched homologous non-tumor tissue from and tumor-adjacent, but cancer-free area was analyzed for expression levels of selected genes associated with the regulation of cell cycle and apoptosis, including BCL2 (HS00608023), TP53 (HS1034249), and the transcription factors FOXA1 (HS00270129), and GATA3 (HS00231122). The PCR reactions were performed on the Eco Real-Time PCR System (Illumina). The reaction was performed in a 5µl mixture consisting of 2.5µl Maxi-ma Probe/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific), 0.25µl of each primer – Taq-Man®Gene Expression Assay (Applied Biosystems), 0.5µl of cDNA and the rest of the reaction volume was adjusted with water. The thermal cycling conditions were composed of 50 ºC for 2 min followed by an initial denaturation step at 95 ºC for 10 min and 45 cycles at 95 ºC for 15s, and 60 ºC for 1 min. Forty-five amplification cycles were applied, and the cycle threshold (Ct) values of the genes of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample were estimated as the mean value of the triplicate measurements. Ct values were then

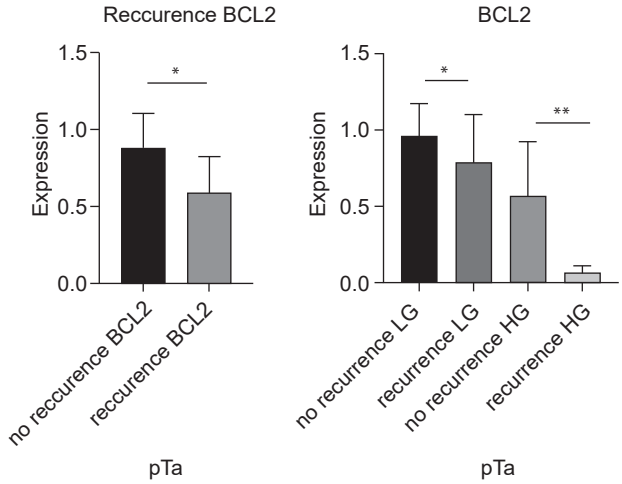

Fig. 1. Expression levels of BCL2. a) Comparing recurrence to newly diagnosed pTa BC. b) Stratified by Grade.

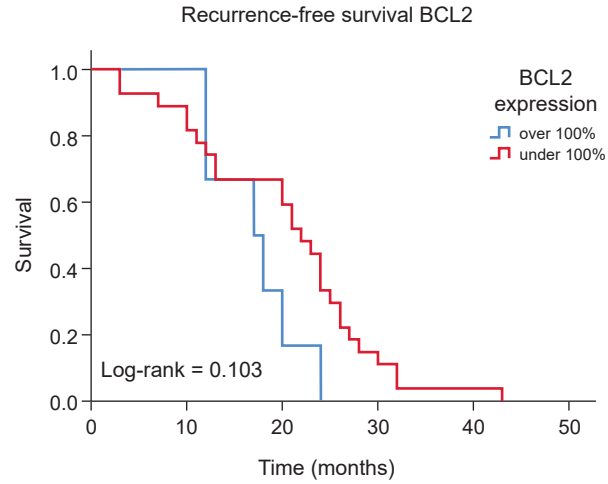

Fig. 2. Kaplan–Meier survival curve of recurrence-free survival of BC patients stratified by expression levels of BCL2.

normalized against the mean expression levels of GAPDH. The relative quantification (RQ) of the gene was determined using the ΔΔCT method.

Statistical analysis

The Bonferroni-Holm Method was used to compare RQs of BCL2, TP53, FOXA1, and GATA3 in pTa BC and cancer recurrence. Gene expressions were compared to publicly published expressions in TNMplot: differential gene expression analysis in Tumor, Normal, and Metastatic tissues (tnmplot.com). In the non-tumor tissue, the individual gene expressions represented RQ = 1 or 100% of the expected expression to which the expressions in cancer tissue were compared. Error bars in graphs represent standard deviation (SD). The recurrence-free survival (RFS) stratified by gene expression is presented by the Kaplan–Meier survival curve using the follow-up time until recurrence counted in months, which were taken as the end points of the Kaplan–Meier survival analyses. The Log-rank test was used to identify the statistical differences. Multivariate Cox regression analysis was used to identify prognostic factors. The results were considered significant if $p<0.05$. The date of the first TUR-BT or first histologically positive test was chosen as the starting point of patients' recurrence-free survival (RFS). Statistical analyses were performed with IBM SPSS Statistics version 26 (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY, USA) and GraphPad Prism version 8 (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA).

Tab. 1. Clinicopathologic data of the study group.

Variable	Stratification	n (%)
Gender	Male	54 (68.4)
	Female	25 (31.6)
Age	Median	68 (42–90)
Grade	Low grade	64 (81.0)
	High grade	15 (19.0)
Recurrence	Yes	33 (41.8)
	No	46 (58.2)
RFS*	Median	21 (3–43)

* RFS – recurrence-free survival

Results

The study group contained 79 patients with histopathologically confirmed pTa stage of BC. The RQs of BCL2, TP53, FOXA1, and GATA3 were analyzed in association with cancer recurrence and then stratified by tumor grade. The clinicopathological and demographic characteristics of the study group are summarized in Table 1. One patient died during the follow-up period. 15 HG patients received adjuvant intravesical therapy with bacillus Calmette-Guérin (BCG). 10 out of these patients experienced recurrence, indicating HG was an independent factor, while the use of BCG did not affect RFS (OR=0.426, 0.179–1.017). Radiotherapy and chemotherapy were not indicated in any of these patients. The comparison between this study and expressions documented in tnmplot.com is seen in Table 2.

The expression levels of BCL2 as seen in Figure 1a were lower in patients with recurrence, however not statistically significant. Similarly, in Figure 1b, a comparison of LG and HG tumors, the expression of BCL2 gradually lowers with rising tumor grade with a significant loss of expression in the HG recurrence group ($p<0.01$). This causes dysregulation in bcl-2 protein family production and damages apoptosis regulation. RFS did not prove statistically significant in association with BCL2 expression. In

Tab. 2. Comparison of expressions with tnmplot.com.

Gene	This study	Tnmplot.com
BCL2	Underexpression	Underexpressed in BC
TP53	Overexpression	Overexpressed in BC
FOXA1	Underexpression	No significant change
GATA3	Overexpression	Overexpressed in BC

the Kaplan–Meier survival curve (Fig. 2) we can see that patients with initially lower expression of BCL2 exhibited somewhat longer RFS (Log-rank=0.103).

Expression levels of TP53 presented in Figures 3a and 3b had the tendency to rise with tumor recurrence independently of the initial grade ($p<0.01$). RFS in Figure 4 did not prove statistically significant in association with TP53 expression (Log-rank=0.928).

FOXA1 did prove statistically significant in LG association to recurrence and RFS. Expression levels of FOXA1 presented in Figures 5a and 5b point to a decrease of gene expression in the recurrence of LG tumors which translates to lower protein production and dysregulation in normal urothelial differentiation. However, this did not prove statistically significant in the case of HG tumors and RFS (Fig. 6).

In Figures 7a and 7b, the expression levels of GATA3 showed a significant ($p<0.01$) decrease in both LG and HG tumors in subjects with recurrence, while the initial expression levels of HG tumors were exponentially higher than those of LG. In Figure 8, the expression of GATA3 did prove statistically significant in association with RFS (Log-rank = 0.015) with similar results to BCL2, where subjects with initially low expression tended to have longer RFS. This finding complements the difference between HG versus LG expressions shown in Figure 7b.

Discussion

In 2006, the GUCG published a scoring system, later reused by Suh et al 2021 and risk tables based on the WHO 1973 classification, in which patients with G1/G2 pTa tumors who received chemotherapy are stratified into 3 risk groups for recurrence (33). These risk groups are based on the history of recurrences, intravesical treatment, tumor grade, number of tumors, and adjuvant chemotherapy (37, 34). Currently, these are the only systems used (acknowledged by EAU) to predict recurrence in NMIBC. It is

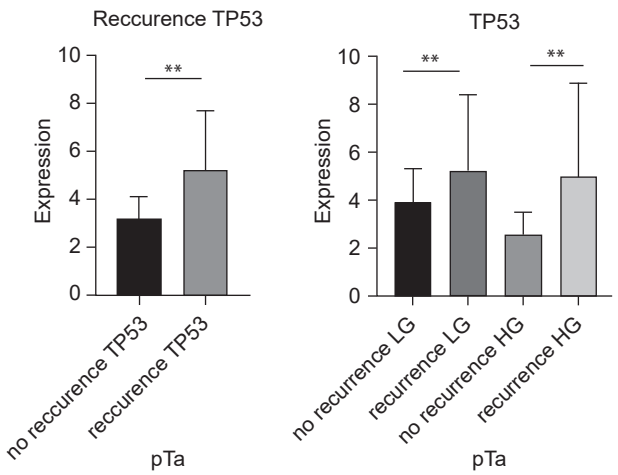

Fig. 3. Expression levels of TP53. a) Comparing recurrence to newly diagnosed pTa BC. b) Stratified by Grade.

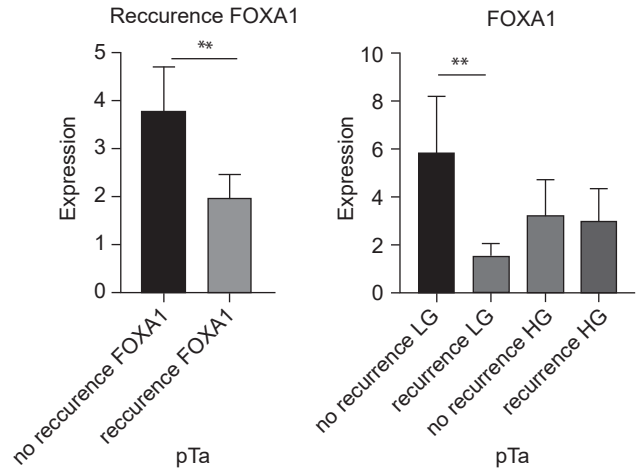

Fig. 5. Expression levels of FOXA1. a) Comparing recurrence to newly diagnosed pTa BC. b) Stratified by Grade.

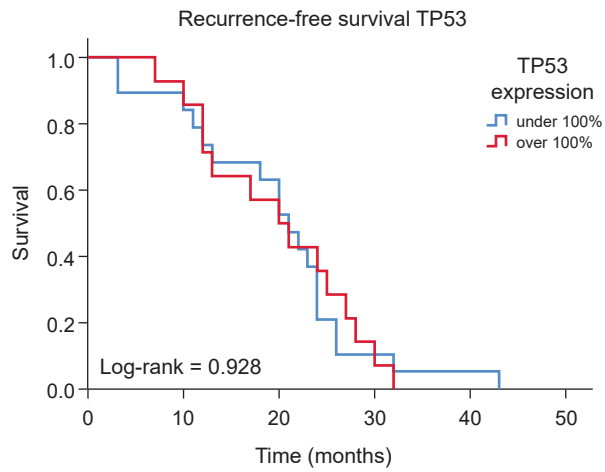

Fig. 4. Kaplan–Meier survival curve of recurrence-free survival of BC patients stratified by expression levels of TP53.

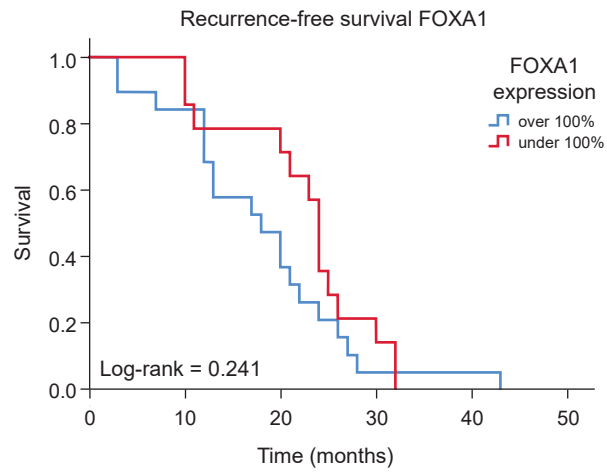

Fig. 6. Kaplan–Meier survival curve of recurrence-free survival of BC patients stratified by expression levels of FOXA1.

obvious that these systems are based on „classic" histopathologic and clinical tumor characteristics, and no molecular signatures are taken into prediction models yet (35–37). However, for MIBC, there was a consensus on molecular classification, where authors classified TP53, FOXA1, and GATA3 (among others) as luminal markers, emphasizing reporting rather biological than clinical classes of BC (38). Robertson et al in their work focused on T1 and higher BC stages, where they successfully identified and characterized expression subtypes of HG stage T1 BC. Also, in 2017, they reported an analysis of 412 MIBCs, in which fifty-eight genes were significantly mutated (39, 40).

To date, the UROMOL study by Lindskrog et al was the most comprehensive multi-omics analysis on NMIBC tumors from 834 subjects. The authors also created an online tool for the classification of independent samples (41).

Our study demonstrates the value of relative expression of BCL2, TP53, FOXA1, and GATA3 in association with the prediction of recurrence in pTa BC. With this different approach to recurrence and RFS, our results provide additional information to studies analyzing molecular markers and the recurrence of NMIBC.

It is obvious that the molecular landscape in NMIBC is based on broad biological heterogeneity, which can lead to unpredictable clinical outcomes in terms of recurrence and progression. Recent work by Piao et al aimed at risk assessments in NMIBC and compared the predictive value of the molecular signature-based subtype predictor (MSP888) and risk calculators based on clinicopathological factors (EORTC, CUETO, and 2021 EAU risk scores) (42). Regarding recurrence, particularly in patients without an intravesical BCG immunotherapy, MSP888 was significantly linked with the risk of disease recurrence and progression (both $p< 0.05$). According to this study, the EORTC, CUETO, and 2021 EAU risk scores showed disappointing results in the estimation of the NMIBC prognosis and recurrence.

The expression of BCL2 was significantly reduced in the BC recurrence samples we studied, thus we assumed an amplification of the apoptotic signaling pathway. We also observed the expression levels to gradually lower with rising tumor grade, until, at a certain stage, many non-detects indicated cessation of expression. Our findings go in hand with a previous study that reported better prognosis in patients with still detectable BCL2 expression compared to non-detects (43). We also managed to associate increased gene expression with the histological grade of bladder tumors, similar to Pollack et al (44). In association with RFS Eimani et al reported shorter RFS in subjects with mRNA expression ratio of Bcl-2/Bax under 1 (45). In our study, we observed a contradictory result, where patients with initially lower (but still detectable) expression of BCL2 in cancer tissue tended to be recurrence-free longer.

According to the study by Kim et al, the variability of TP53 expression correlates with the variability of IHC-detectable p53 protein, which suggests a possible mutation and loss of function in higher-stage tumors, not only in BC, but many types of genitourinary tumors (46, 47). Alterations in TP53 expression were reported to be predictive in response to BCG treatment (48). Overexpression and accumulation of TP53 is then a predictor of poor prognosis

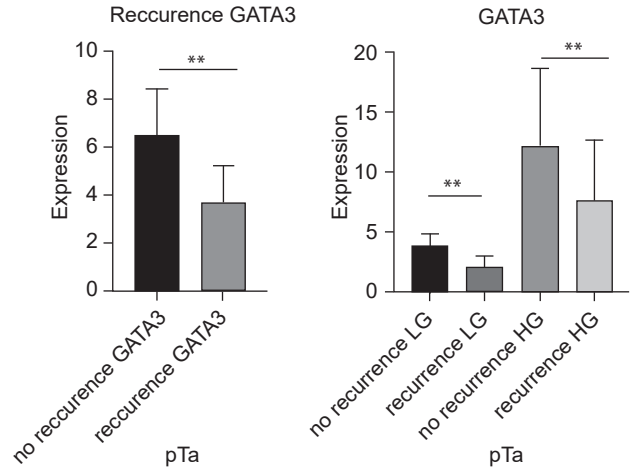

Fig. 7. Expression levels of GATA3. a) Comparing recurrence to newly diagnosed pTa BC. b) Stratified by Grade.

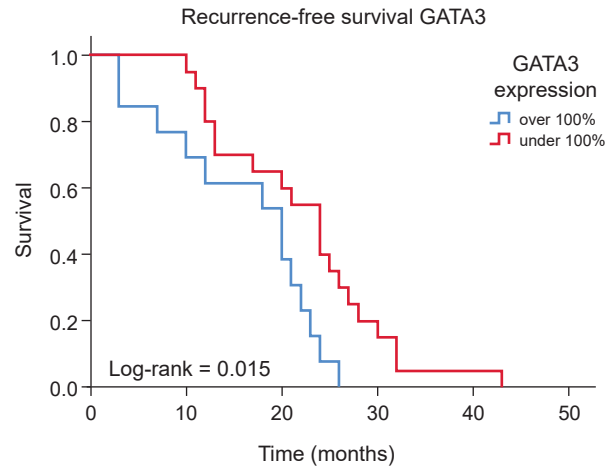

Fig. 8. Kaplan–Meier survival curve of recurrence-free survival of BC patients stratified by expression levels of GATA3.

in advanced-stage bladder tumors (49). TP53 expression has also been found to be associated with disease recurrence (50). Also, a meta-analysis suggested that overexpression of TP53 in NIMBC patients treated with BCG could be associated with RFS (51). Our results correlate with those of previous studies. We observed that increased expression of the TP53 gene is not only present in recurrence generally, but individual grades exhibit a rise in TP53 expression in recurrence.

Reduced expression of the FOXA1 gene was correlated with increasing tumor stage, while complete loss of expression of FOXA1 is associated with a high histological stage of BC (21). In our study, we also observed that reduced expression of the FOXA1 gene correlates with increasing tumor stage. When stratified by grade, only the LG group showed significant differences in expression.

A recent meta-analysis on the association between estrogen receptors and GATA3 in BC reported a significant association of

GATA3-positive samples with higher RFS (52). A recent study reported GATA3 expression to be associated with local recurrence, independent of gender, age, tumor differentiation, and stage in NMIBC (53). We observed a significant increase in GATA3 expression in HG tumors compared to LG which points to a regulatory increase of expression in more severe lesions. In patients with recurrence, expression gradually decreases, which may lead to a complete gene loss in the future, representing the aforementioned poor prognosis (54). In the case of RFS, we observed similar results as in the case of BCL2, where subjects with lower RQ tended to be longer recurrence-free.

The limitations of this study were mainly the small sample size and uneven demographic distribution of respondents. Also, using homologous non-tumor tissue adjacent to the tumor area combined with cancer tissues from the same subject for RT-qPCR relative quantification instead of a quantitative comparison to completely healthy subjects could have affected the expression results. However, obtaining bladder biopsy samples from healthy subjects is very complicated. Choosing specifically only the pTa group of NMIBC could have distorted the results if compared to both pTa and pT1 stages. Even though the study took place during the COVID-19 pandemic, which affected the number of patients in later years, and often complicated follow-ups, only one patient died during the follow-up time.

Conclusions

It is clear that the assessment of the risk of NMIBC recurrence based only on histopathological features is insufficient when considering tumor heterogeneity and various biological landscapes that drive tumor behavior. Several molecular markers were proposed, mainly in combination and the results indicate that the expert societies (EAU, EORTC) will face the challenge of proper consideration and incorporation into guidelines. However, further validation, cost-benefit analysis, and larger prospective trials are still warranted. To our knowledge, this is the first study analyzing the relative quantification of BCL2, TP53, FOXA1, and GATA3 to assess the risk of recurrence in pTa BC. Our results, indicate, that comparing expression levels of these genes in cancer and cancer-free tissue could provide valuable data, as patients with pTa BC recurrence within up to 54 months of follow-up had a significantly higher RQ of TP53, GATA3, and FOXA1 when compared to pTa BC patients without recurrence.

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