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ORIGINAL RESEARCH

The Prognostic Value of FGFR3 Expression in Patients with T1 Non-Muscle Invasive Bladder Cancer

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Purpose: Fibroblast growth factor receptor 3 (FGFR3) alterations are frequent in non-muscle-invasive bladder cancer (NMIBC), although current data regarding the prognostic and therapeutic relevance are inconsistent. We analyzed the prognostic role of FGFR3 mRNA expression in stage T1 NMIBC.

Patients and Methods: The mRNA expression of FGFR3 and cyclin-dependent kinase inhibitor 2A (CDKN2A) was measured by RT-qPCR in 80 patients with stage T1 NMIBC treated with transurethral resection of the bladder and correlated with clinical data and KRT5 and KRT20 expression, used as surrogate markers for basal and luminal subtypes, respectively.

Results: FGFR3 and CDKN2A transcript levels were not correlated. FGFR3 expression was associated with the expression of KRT5 ($p=0.002$) and KRT20 ($p < 0.001$). CDKN2A expression was negatively correlated with KRT5 ($p=0.030$). In Kaplan–Meier analysis and univariable Cox regression analysis, high FGFR3 expression was associated with significantly reduced recurrence-free survival (RFS) (HR=3.78; $p < 0.001$) and improved overall survival (OS) (HR=0.50; $p=0.043$), while high CDKN2A expression was associated with reduced OS (HR=2.34; $p=0.034$). Patient age was the only clinicopathological parameter associated with reduced OS (HR=2.29; $p=0.022$). No parameter was an independent prognostic factor in multivariable analysis. Next, we stratified the patients depending on their lineage differentiation. In univariable analysis, the prognostic effect of FGFR3 and CDKN2A was observed primarily in patients demonstrating high expression of KRT5 or KRT20, whereas high FGFR3 expression was associated with significantly reduced RFS, irrespective of instillation therapy.

Conclusion: Stage T1 NMIBC patients with high FGFR3 expression show shorter RFS but better OS than patients with low FGFR3 expression.

Keywords: biomarker, CDKN2A, FGFR3, NMIBC, prognosis

Introduction

Urothelial carcinoma of the bladder (UCB) is the 11th most common cancer worldwide.[1] Approximately 75% of newly diagnosed UCBs are non-muscle-invasive bladder cancers (NMIBC), which include stages Ta, T1 and carcinoma in situ (CIS). NMIBCs are usually treated with a bladder-sparing approach comprising transurethral resection of the bladder (TUR-B) followed by regular cystoscopies. As an adjuvant treatment, instillation therapies of mitomycin or Bacillus Calmette-Guérin (BCG) can be administered, depending on tumor status.[2] High rates of recurrence and progression to muscle-invasive bladder cancer (MIBC)

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necessitate frequent follow-up examinations, which pose a heavy burden for the patient as well as the health care system.[3,4] Although there have been some improvements in the treatment of metastatic disease in recent years, with the introduction of numerous checkpoint inhibitors as well as the accelerated approval of erdafitinib by the Food and Drug Administration (FDA), no new diagnostic methods or therapeutic options have been established for NMIBC.[5,6] The lack of innovation is especially critical for patients with stage T1 NMIBC, as 70% experience a disease recurrence after BCG, while 33% of these patients even progress to MIBC.[7] Patients classified as having the highest risk of NMIBC according to the criteria developed by the European Organization for Research and Treatment of Cancer (EORTC) should be considered for an early cystectomy.[8] The investigation and clinical implementation of novel molecular markers beyond the established clinicopathological characteristics could be a helpful addition to better distinguish patients with stage T1 NMIBC suitable either for a bladder-sparing approach or early cystectomy.

Aberrations such as mutations or overexpression of the fibroblast growth factor receptor 3 (FGFR3) are very frequent in UCB with mutations occurring in approximately 40% of patients, of whom 70–80% have low-grade NMIBC, which allows ligand-independent dimerization, phosphorylation and downstream signaling.[9] Given the high frequency of FGFR3 mutations in urothelial papilloma and hyperplasia, both of which are considered precursors of papillary UCB, FGFR3 mutations supposedly occur early in the process of tumor development.[10] An association between FGFR3 mutations and lower stage and grade has been shown in several studies,[11–13] which is also the case for patients with FGFR3 overexpression.[14,15] Moreover, FGFR3 overexpression was previously associated with increased FGFR3 mutation rates. However, approximately 40% of UCBs with FGFR3 overexpression do not harbor any FGFR3 mutations, many of which are MIBCs.[16]

Nevertheless, the prognostic relevance of FGFR3 with regard to survival in NMIBC remains unclear. In terms of cancer recurrence, several studies have demonstrated an association with reduced recurrence-free survival (RFS),[17] while others have found FGFR3 mutations to be associated with lower recurrence rates.[11] Regarding the association with progression-free survival (PFS), the results are conflicting as well. For instance, Burger et al found FGFR3 mutations to be associated with longer PFS,[13] unlike Hernández et al, who found no prognostic relevance of FGFR3 mutations.[18] These conflicting results are especially apparent in stage T1 NMIBC, which may be due to the many molecular similarities this tumor entity shares with stage Ta NMIBC as well as MIBC.[9,19] The combination of FGFR3 expression with other markers might be necessary to improve the predictive value of FGFR3 expression in stage T1 NMIBC.

To overcome the known confinements of immunohistochemistry, we recently investigated the mRNA expression of FGFR3 with reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assessment in a large cohort of 296 patients with stage T1 NMIBC and found an association of high FGFR3 expression with improved PFS.[20] In addition to FGFR3, we also measured the mRNA expression of cyclin-dependent kinase inhibitor 2A (CDKN2A), which encodes the tumor suppressor protein p16.[21] In other studies, a loss of heterozygosity in the region 9p of chromosome 9, and thereby the deletion of CDKN2A, has been associated with a higher grade and worse outcome.[22,23] Interactions between CDKN2A and FGFR3 have previously been suggested by other investigators.[24] In our previous study, high mRNA expression of CDKN2A was associated with reduced PFS, with the subgroup of patients with high CDKN2A and low FGFR3 expression displaying the worst PFS.[20]

The goal of the current study was to validate the prognostic relevance of the mRNA expression of FGFR3 and CDKN2A within a new independent cohort of patients with stage T1 NMIBC in order to allow for a future implementation into the diagnostics and therapy in a daily clinical routine.

Patients and Methods

Patient Population

In total, the clinical and histopathological data of 80 patients treated with TUR-B at the Department of Urology and Pediatric Urology of the University Hospital Erlangen between 2000 and 2015 were retrospectively analyzed. Only patients initially diagnosed with stage T1 NMIBC and treated with a bladder-sparing approach were included in this study. All patients received a Re-TUR-B within six to eight weeks after the initial TUR-B. Tumor tissue slices of all patients were evaluated for pathological stage according to the 2010 TNM classification and graded according to the common grading systems (WHO 1973, WHO 2016) by two experienced uropathologists (ME, AH). All specimens contained at

least 50% tumor cells. All patients gave informed consent. All procedures were performed in accordance with the ethical standards established in the 1975 Declaration of Helsinki. The study was approved by the Ethics Committee of the University Hospital Erlangen (No. 3755 and No. 296_18 Bc). Recurrence was defined as the reappearance of UCB, either NMIBC or MIBC, while progression was defined as the progression to MIBC or metastatic disease.

Assessment of mRNA Expression by RT-qPCR

Tumor specimens were assessed by RT-qPCR as previously described.[20] In short, after extraction from a single 10-µm curl of FFPE tissue, the RNA was then processed according to a commercially available bead-based extraction method (Xtract kit; STRATIFYER Molecular Pathology GmbH, Cologne, Germany). RNA was eluted with 100 µL of elution buffer. DNA was digested, and RNA eluates were then stored at −80°C until use.

The mRNA expression levels of FGFR3 and CDKN2A were assessed, in addition to the keratins KRT5 and KRT20 as surrogate markers for basal and luminal markers of UCB, similar to our previous studies.[25,26] Furthermore, mRNA expression of the proliferation marker Ki-67 (MKI67) was measured. Calmodulin-2 (CALM2) and ß2-microglobulin (B2M) were used as reference genes. The mRNA expression was determined by a one-step reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)-based assessment, which involves the reverse transcription of RNA and subsequent amplification of cDNA executed in a one-step reaction. Each patient sample or control was analyzed in duplicate using the Invitrogen SuperScript III RT-qPCR system (Thermo Fisher Scientific, Waltham, MA, USA) and gene-specific primer-probe combinations (STRATIFYER Molecular Pathology). Each patient's sample was analyzed in duplicate on an ABI Step One PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions. Gene expression was quantified with a modification of the method by Schmittgen and Livak by calculating 40-ΔCt, whereas ΔCt was calculated as the difference in Ct between the test gene and the mean of the reference genes.[20]

Statistical Methods

Correlations between the mRNA expressions of FGFR3, CDKN2A, KRT5, KRT20, and MKI67 were calculated using Spearman's bivariate correlation. Optimized cut-off values for each marker with regard to survival were defined using Youden's index on the receiver operating characteristic (ROC) curve. The date of the first TUR-B was defined as the common time point zero for retrospective survival analysis. The associations of clinicopathological markers (Grade WHO 1973, concomitant carcinoma in situ (CIS), instillation therapy, gender, age) and mRNA expression of the molecular markers with RFS, PFS, overall survival (OS), and cancer-specific survival (CSS) were determined by univariable (Kaplan–Meier analysis and Cox regression hazard models) and multivariable analyses (Cox regression hazard models). All tests were two-sided, and p-values <0.05 were considered statistically significant. Statistical analyses were performed with the SPSS 21.0 software package (SPSS Inc., Chicago, IL, USA) and R V3.2.1 (The R foundation for statistical computing, Vienna, Austria).

Results

Patient Population

The clinicopathological characteristics of the cohort are summarized in Table 1. Three quarters of patients were males. The median age at diagnosis was 71 years (46–97), and the median follow-up was 62 months (range 0–189 months).

Almost half of the patients (51.2%) received an adjuvant instillation therapy with either mitomycin or BCG, which is comparable to real-world data showing an application of postoperative instillation therapy in 29–65% of patients with high-risk NMIBC.[27,28]

Correlation of the mRNA Expression of FGFR3, CDKN2A, KRT5, KRT20, and MKI67 with Each Other and with Clinicopathological Parameters

Nonparametric Spearman's rank test revealed no correlation between the mRNA expression of FGFR3 and CDKN2A. CDKN2A showed a significant negative correlation with KRT5 (r_s= −0.24; p=0.030), ie, the basal subtype. In contrast, there was no correlation with KRT20, ie, the luminal subtype. FGFR3 was significantly associated with both KRT5 (r_s=0.35; p=0.002) and KRT20 (r_s=0.39; p < 0.001). Neither FGFR3 nor CDKN2A was significantly associated with MKI67. High FGFR3 expression was negatively correlated with tumor grade according to the WHO 1973 classification (r_s= −0.33; p < 0.001) and concomitant CIS (r_s= −0.33; p < 0.001). There was no

Table 1 Patient Cohort (IQR=Interquartile Range)

		n (%)
Total cohort		80 (100%)
Gender	Male Female	61 (76.3) 19 (23.7)
Median age years (IQR)	71 (46–97)	
Median follow-up months (IQR)	62.0 (0.0–189.0)	
Tumor grade (WHO 2016)	Low grade High grade	2 (2.5) 78 (97.5)
Tumor grade (WHO 1973)	G2 G3	32 (40.0) 48 (60.0)
Lymphovascular invasion	Yes No/NA	2 (2.5) 78 (97.5)
Concomitant CIS	Yes No/NA	28 (35.0) 52 (65.0)
Adjuvant instillation	Yes No	41 (51.2) 39 (48.8)
Median follow-up months (IQR)	62.0 (0.0–189.0)	
Recurrence-free survival (RFS)	Recurrence No recurrence	41 (51.2) 39 (48.8)
Progression-free survival (PFS)	Progression No progression	6 (7.5) 74 (92.5)
Cancer-specific survival (CSS)	Cancer-specific death Others	16 (20.0) 64 (80.0)
Overall survival (OS)	Death Alive	36 (45.0) 44 (55.0)

Abbreviations: IQR, interquartile range; CIS, carcinoma in situ; WHO, World Health Organization; NA, not available.

association between FGFR3 or CDKN2A and age, gender, or instillation therapy.

Association of mRNA Expression of FGFR3, CDKN2A and Clinicopathological Parameters with Survival

We used receiver-operating characteristic (ROC) analyses to determine the optimal cut-off values for FGFR3 and CDKN2A with regard to survival (Table 2). For each clinical endpoint (OS, CSS, RFS, PFS) an optimal cut-off value was determined by the Youden index and applied in Kaplan Meier analyses (log rank test). Kaplan–Meier analysis showed high mRNA expression of FGFR3 to be significantly associated with reduced RFS ($p < 0.001$) and improved OS (p=0.039), in addition to showing a nonsignificant trend towards improved CSS (p=0.067). Interestingly, high FGFR3 expression was also associated with prolonged PFS (p=0.037); however, given that only six patients (7.5%) had a documented time of progression despite 16 patients suffering a cancer-specific death, we excluded PFS from any further survival analyses.

High expression of CDKN2A was significantly associated with reduced OS (p=0.029) and showed a nonsignificant trend towards reduced CSS (p=0.057). There was no association between mRNA expression of CDKN2A and RFS or PFS.

In the univariable Cox regression analysis, high FGFR3 expression was associated with a significantly

Table 2 Results of Kaplan–Meier Analysis (Log Rank Test): Cut-off Values for FGFR3 and CDKN2A with Regard to Recurrence-Free (RFS), Progression-Free (PFS), Cancer-Specific (CSS) and Overall Survival (OS) for the Total Cohort (n=80)

Marker	Survival	Cut-off Value (40-ΔCt)	n High (%)	n Low (%)	p-value
FGFR3	RFS	42.1	12 (15.0)	68 (85.0)	**<0.001**
	PFS	38.7	26 (32.5)	54 (67.5)	**0.038**
	CSS	39.6	35 (43.8)	45 (56.2)	0.067
	OS	39.8	37 (46.2)	43 (53.8)	**0.039**
CDKN2A	RFS	30.8	12 (15.0)	68 (85.0)	0.285
	PFS	35.6	58 (72.5)	22 (27.5)	0.180
	CSS	35.8	61 (76.2)	19 (23.8)	0.057
	OS	33.4	32 (40.0)	48 (60.0)	**0.029**

Note: Significant values are in bold.

increased risk of recurrence (hazard ratio (HR)=3.78; $p < 0.001$) and an improved chance for prolonged OS (HR=0.50; p=0.043). High CDKN2A was associated with a higher risk of reduced OS (HR=2.34; p=0.034).

Of the clinicopathological parameters, only age was associated with shorter CSS (HR=3.44; p=0.034) and OS (HR=2.29; p=0.022) in the univariable Cox regression analysis. Tumor grade; concomitant CIS; instillation therapy; gender; and the molecular parameters MKI67, KRT5, and KRT20 were not associated with prognosis (OS, CSS, RFS) and therefore were not included in further multivariable Cox regression analyses.

The multivariable analysis of OS adjusted for age and the expressions of FGFR3 and CDKN2A revealed that none of the parameters were independent prognostic factors. Given that FGFR3 expression was the only significant prognostic marker for RFS, no multivariable analysis was conducted for FGFR3.

Association of FGFR3 and CDKN2A mRNA Expression with RFS and OS Stratified by Clinicopathological Parameters or mRNA Expression

Having found an association between RFS and mRNA levels of FGFR3 as well as between RFS and both FGFR3 and CDKN2A in the total cohort, we sought to analyze the prognostic relevance of these two markers within different patient subgroups defined by clinicopathological parameters.

Stratification by Age

Using the median age of 71 years as a cut-off to define the two age groups (≤71 vs >71 years), patients aged ≤71 years who had NMIBC with high FGFR3 expression demonstrated significantly improved OS when compared to patients with low FGFR3 expression (p=0.007; Log rank test) (Figure 1). In the univariable Cox regression analysis, patients aged ≤71 years with a high FGFR3 expression had a significantly reduced risk of death (HR=0.22; p=0.013) (Table 3). In patients older than 71 years, FGFR3 expression had no effect on OS. With regard to RFS, high FGFR3 expression was significantly associated with a shorter time to recurrence irrespective of the patients' age (≤71 years: p=0.004; >71 years: p=0.010; Log rank test). The risk of experiencing a recurrence was also significantly increased with high FGFR3 expression in both age groups (≤71 years HR=4.87; p=0.008; >71 years HR=3.08; p=0.015) (Table 3) in the univariable Cox regression analysis.

CDKN2A demonstrated no association with OS when the cohort was separated into the two age groups.

Stratification by KRT5 and KRT20 Expression

Both KRT5 and KRT20 mRNA expressions were used as surrogate markers to define basal and luminal subtypes of UCB by analogy to previous studies.[25] Median expression levels were used as cut-offs to subdivide patients into low/high KRT5 (≤36.78 vs >36.78) or low/high KRT20 (≤37.47 vs >37.47) mRNA expression.

As mentioned above, FGFR3 was associated with both markers. Patients with high expression of KRT5 were associated with reduced RFS ($p < 0.001$; Log rank test) and improved OS (p=0.023, Log rank test) when FGFR3 expression was high. In these patients, FGFR3 was also associated with an increased risk of recurrence (HR=4.92; $p < 0.001$) (Table 3) but a better chance of improved OS (HR=0.27; p=0.032) in the univariable Cox regression

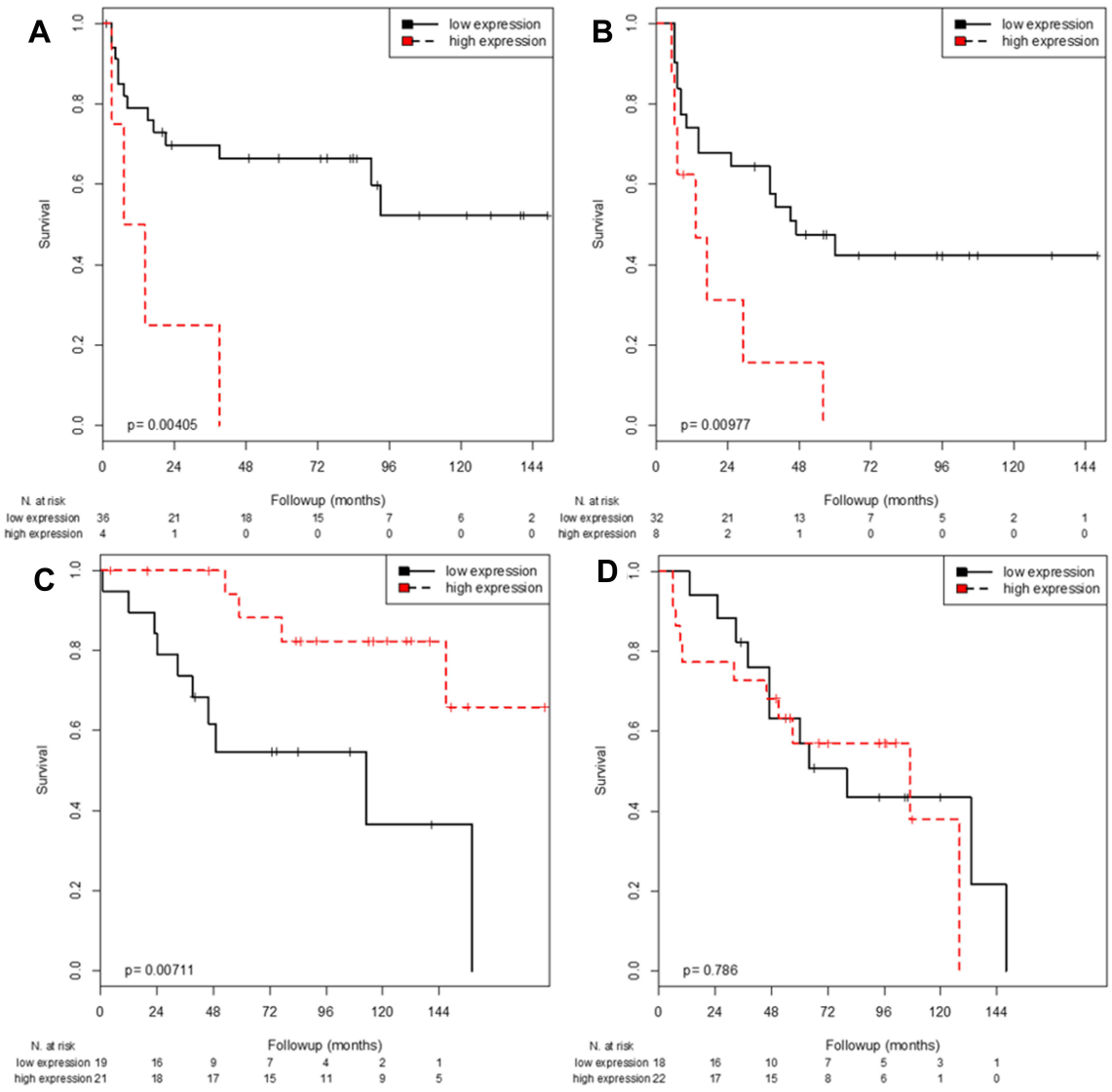

Figure 1 Kaplan-Meier analysis of FGFR3 mRNA expression regarding RFS in patients ≤71 years (**A**) and patients >71 years (**B**) as well as regarding OS in patients ≤71 years (**C**) and patients >71 years (**D**).

analysis. In patients with low KRT5 expression, FGFR3 expression showed no effect on survival. In patients with high KRT20 expression, high FGFR3 expression was associated with reduced RFS (p=0.004; Log rank test; HR=3.43, p=0.007; univariable Cox regression analysis) (Table 3) but not OS.

While CDKN2A showed no positive association with either KRT5 or KRT20 in the total cohort, patients with high expression of both markers showed a significantly reduced OS when CDKN2A expression was high (KRT5 $p < 0.001$; KRT20 p=0.039) (Figure 2). CDKN2A was an independent prognostic marker for reduced OS in patients with high expression of KRT5 (HR=9.06; p=0.005) and KRT20 (HR=3.03; p=0.049) (Table 3). No effect of CDKN2A on OS was observed when the mRNA expression of KRT5 and KRT20 was low.

Combined, the current results indicate a prognostic effect of high FGFR3 or CDKN2A expression, especially in patients also presenting with either a basal or luminal-like subtype, while no prognostic relevance was seen in patients who could not be assigned to either of these subtypes.

Stratification by MKI67 Expression

MKI67 is a prominent marker associated with the proliferation activity of tumor cells.[29] As with KRT5 and KRT20, patients were divided into groups with low or high mRNA expression of MKI67 using the median

expression level (≤33.10 vs >33.10). This way, patients with both low and high MKI67 expression were associated with significantly reduced RFS (MKI67 low p=0.013; MKI67 high p=0.010; Log rank test) when FGFR3 expression was high, and there was an increased risk of recurrence in both groups (MKI67 low: HR=4.59; p=0.022; MKI67 high: HR=2.99; p=0.014; univariable Cox regression analysis) (Table 3), unlike patients with low expression of FGFR3. There was no association with OS irrespective of MKI67 expression or the expression of

Table 3 Univariable Cox Regression Analysis for Stratification by Clinicopathological or Molecular Parameters: The Association of FGFR3 and CDKN2A mRNA with OS and RFS

Parameter by Stratification	Overall Survival			Recurrence-Free Survival		
	N	HR	*p*	N	HR	*p*
Age ≤ 71 years						
FGFR3 high vs low	21 vs 19	0.22	**0.013**	4 vs 36	4.87	**0.008**
CDKN2A high vs low	22 vs 18	2.94	0.098	33 vs 7	3.88	0.189
Age > 71 years						
FGFR3 high vs low	22 vs 18	1.13	0.790	8 vs 32	3.08	**0.015**
CDKN2A high vs low	26 vs 14	1.50	0.436	35 vs 5	1.01	0.988
KRT5 high						
FGFR3 high vs low	25 vs 15	0.27	**0.032**	9 vs 31	4.92	**<0.001**
CDKN2A high vs low	21 vs 19	9.06	**0.005**	32 vs 8	1.71	0.388
KRT5 low						
FGFR3 high vs low	18 vs 22	0.80	0.630	3 vs 37	2.78	0.183
FCDKN2A high vs low	27 vs 13	0.76	0.580	36 vs 4	1.95	0.518
KRT20 high						
FGFR3 high vs low	26 vs 14	0.43	0.071	10 vs 30	3.43	**0.007**
CDKN2A high vs low	23 vs 17	3.03	**0.049**	36 vs 4	1.48	0.596
KRT20 low						
FGFR3 high vs low	17 vs 23	0.52	0.226	2 vs 38	2.90	0.160
CDKN2A high vs low	25 vs 15	1.77	0.324	32 vs 8	1.90	0.391
MKI67 high						
FGFR3 high vs low	25 vs 15	0.43	0.058	8 vs 32	2.99	**0.014**
CDKN2A high vs low	24 vs 16	2.13	0.141	35 vs 5	2.06	0.330
MKI67 low						
FGFR3 high vs low	18 vs 22	0.40	0.129	4 vs 36	4.59	**0.022**
CDKN2A high vs low	24 vs 16	2.46	0.164	33 vs 7	1.34	0.700
Instillation						
FGFR3 high vs low	21 vs 20	0.35	0.061	6 vs 35	2.61	**0.047**
CDKN2A high vs low	22 vs 19	2.67	0.092	35 vs 6	1.62	0.434
No instillation						
FGFR3 high vs low	22 vs 17	0.81	0.623	6 vs 33	5.44	**0.003**
CDKN2A high vs low	26 vs 13	1.93	0.241	33 vs 6	2.18	0.454

Note: Significant values are in bold.
Abbreviation: HR, hazard ratio.

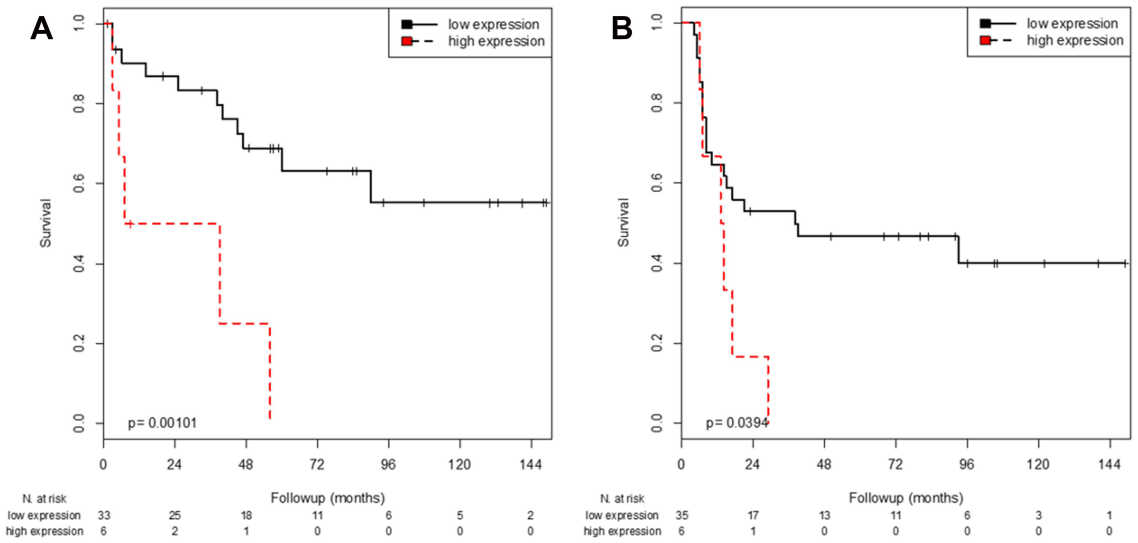

Figure 2 Kaplan-Meier analysis of FGFR3 mRNA expression regarding RFS in patients who received no postoperative instillation (**A**) and patients with postoperative instillation (**B**).

either FGFR3 or CDKN2A. These data suggest that MKI67 expression plays no relevant role in the increased risk of recurrence for patients with high FGFR3 expression.

Stratification by Instillation

Independent of intravesical instillation therapy, patients with high expression of FGFR3 showed a shorter RFS (no instillation: p=0.001; installation: p=0.039; Log rank test) (Figure 2) and had an increased risk of recurrence. Nevertheless, the risk was higher for patients not receiving an instillation therapy (no instillation: HR=5.44, p=0.003; instillation: HR=2.61, p=0.047; univariable Cox regression analysis) (Table 3). There was no effect of instillation therapy on OS irrespective of FGFR3 or CDKN2A expression.

Discussion

Aberrations of FGFR3 are regarded as changes in one of the major pathways in the carcinogenesis of UCB.[30] Consequently, the role and effect of FGFR3 in UCB have been the focus of multiple studies.[12,13,21,31] Most studies have found that FGFR3 alterations, such as mutations and protein overexpression, are associated with lower stage and grade and improved outcome, although the association with survival has not been consistent.[11,13,30,32] For instance, initial analyses by van Rhijn et al demonstrated FGFR3 mutations to be associated with prolonged RFS and CSS.[17,30] By contrast, Hernández et al found FGFR3 mutations to be associated with increased recurrence rates, but only in stage TaG1 NMIBC.[11] Other studies found no association with recurrence, progression or CSS.[11,33,34] Burger et al analyzed 221 patients with NMIBC and showed that FGFR3 mutations were associated with favorable PFS, especially in high-grade and stage T1 NMIBC.[13] This is in line with our previous results showing that FGFR3 mRNA overexpression is associated with improved PFS in 296 patients with stage T1 NMIBC as well as with our current results.[20] The improved outcome is also reflected in the improved OS demonstrated in our current study; however, this is only relevant in younger patients, suggesting that factors other than FGFR3 expression are more substantial in elderly patients. Higher patient age has generally been associated with reduced RFS in a cohort with primary CIS.[35] However, in our cohort of stage pT1 patients, we were not able to detect such an association. Instead, only high FGFR expression was accompanied by reduced RFS, implicating recurring but not very aggressive cancers irrespective of patients' age. Recently, a meta-analysis showed that both FGFR3 mutation and protein overexpression were significantly associated with RFS, PFS, CSS, and overall survival in NMIBC.[36]

While the overall benign effect of FGFR3 mutations in UCB is widely assumed, several studies have examined additional alterations or mutations to improve the stratification of patients with an increased risk of progression. Homozygous deletions of 9p21.3, including the

CDKN2A gene, have been detected by array-based comparative genomic hybridization and/or RT-qPCR in 22% (9/41) of bladder cancer patients.[37] Several groups have investigated FGFR3 mutation in combination with a loss of heterozygosity in the 9p region of chromosome 9, which leads to a deletion of CDKN2A and decreased expression of the tumor suppressor protein p16, consequently promoting tumor progression.[38,39] Ploussard et al investigated 58 patients with NMIBC for their CDKN2A and FGFR3 status.[24] In patients who retained heterozygosity on chromosome 9, FGFR mutational status was not predictive of recurrence or progression to MIBC. On the other hand, FGFR3 mutational status was strongly associated with outcomes in patients with a loss of heterozygosity, with patients with wild-type FGFR3 exhibiting a higher risk for recurrence and progression than patients with FGFR3 mutations.[24] Rebouissou et al found homozygous deletions of CDKN2A to be more frequent in patients with FGFR3-mutated UCB.[39] By analyzing 19 patients with NMIBC and grade heterogeneity, Downes et al found that NMIBCs with FGFR3 mutations demonstrated homozygous deletions of CDKN2A in particular in the low-grade regions of the tumor, suggesting that a loss of CDKN2A precedes grade progression.[40]

Drawing conclusions from these studies, a worse outcome might be expected in patients with reduced CDKN2A expression. Interestingly, our previous study showed high mRNA expression of CDKN2A to be associated with reduced PFS, with the subgroup of patients with high CDKN2A and low FGFR3 expression displaying the worst PFS.[20] The negative effect of high CDKN2A expression in stage T1 NMIBC is in line with our current results, where we found high CDKN2A to be significantly associated with reduced OS and strongly trending towards reduced CSS. In a previous analysis of CDKN2A mRNA expression in patients with MIBC, high expression was associated with reduced RFS and CSS.[41] Moreover, in MIBC, very high or very low CDKN2A expression can be a predictor of worse survival.[42] Previous gene expression analyses displayed distinct mRNA expression patterns for stage Ta NMIBC and MIBC, with stage T1 NMIBC showing signatures of either one or the other.[43] Concerning CDKN2A expression, stage T1 NMIBC might resemble stage Ta NMIBC more than MIBC.

However, it is still unclear why increased CDKN2A mRNA expression appears to be mostly associated with a poor prognosis, whereas CDKN2A is considered to be a tumor suppressor gene.[44] Point mutations in CDKN2A do not play a role in UCB; however, CDKN2A functions upstream of the tumor suppressor RB1, and its expression is related to TP53 expression.[45,46] Mutations in TP53 or RB1 can attenuate the effect of CDKN2A. In The Cancer Genome Atlas (TCGA) data TP53 mutations were observed in 49% and RB1 mutations in 13% of NMIBC patients, and even higher rates have been described by Meeks et al.[47,48] However, this is controversial, since Heedegard et al identified only 8% and 7% mutations in the TP53 and RB1 genes, respectively, in their NMIBC cohort.[49] Furthermore, the relationship between CDKN2A and FGFR3 remains unclear. Al-Khalaf et al showed that CDKN2A can upregulate the expression of several genes involved in cell proliferation, such as fibroblast growth factor receptor 1 (FGFR1), cyclin D1 (CCND1) and E2F1 transcription factor 1 (E2F1). In this way, E2F1 mediates the p16-dependent regulation of several pro- and anti-apoptotic proteins.[50] However, a direct relationship has not yet been shown between CDKN2A and FGFR3. Interestingly, CDKN2A can positively regulate the senescence-associated microRNAs miR-26b, −181a, −210 and −424.[51] An in silico prediction program (TargetScan: http://www.targetscan.org/cgi-bin/targetscan/vert_72/) predicts that miR-181-5p and miR-424-5p can negatively regulate FGFR3. However, further research is necessary to study the possible relationship between CDKN2A and FGFR3.

Regarding the association of FGFR3 with molecular subtypes, the currently available data do not allow for final conclusions. Sjödahl et al showed a high FGFR3 protein expression in the urobasal A subtype,[52] which was in concordance with our previous results showing a correlation of FGFR3 mRNA expression with KRT5.[20] Hurst and Knowles found FGFR3 alterations mainly in the luminal-papillary subtype of MIBC, which is associated with the best overall survival.[53] In the present study, FGFR3 mRNA expression was significantly associated with both KRT5 and KRT20, suggesting that high FGFR3 expression cannot be generally assigned to either the basal or luminal subtype. This may also be due to the special nature of T1 NMIBC, as it can exhibit molecular signatures of either NMIBC or MIBC.[9,43] Intriguingly, when stratifying patients by their KRT5 and KRT20 expression, a prognostic effect of FGFR3 and CDKN2A was observed only when one of the keratins was expressed at high levels. This is indicative of an association of FGFR3 and CDKN2A with either a basal or luminal-like

subtype, unlike other possible subtypes that do not express KRT5 or KRT20.

In several studies reporting on the response to FGFR inhibitors (in MIBC), complete response rates, disease control rates, and overall response rate of 0% to 8%, 59.3% to 64.2%, and 40% were reported for dovitinib, infigratinib, and erdafitinib, respectively.[36] However, the therapeutic consequences of FGFR3 inhibitors in NMIBC remain disputed. Although FGFR3 mutations are mainly associated with NMIBC, no targeted therapies have been approved yet. Our current results demonstrate reduced RFS but prolonged OS in stage T1 NMIBC with high FGFR3 expression, indicating less aggressive but frequently recurring tumors that might benefit from therapies that reduce recurrence rates. Interestingly, while patients with high FGFR3 expression had significantly shorter RFS than patients with low FGFR3 expression, regardless of instillation therapies, the risk of recurrence was five-fold higher when no instillation was applied compared to only approximately two-fold with instillation therapy. These data suggest that patients with high FGFR3 expression still might benefit from a conventional intravesical instillation therapy, which might be improved in combination with targeted therapies. Thus far, however, the sole implementation of anti-FGFR3 therapies in NMIBC has not been successful. In a recent Phase II trial, patients with NMIBC unresponsive to BCG received oral dovitinib.[54] While pharmacodynamically active dovitinib concentrations were observed in urothelial tissues in all patients, over 90% of patients showed no response to therapy, with all patients experiencing at least one grade 3 or 4 toxicity. Currently, ClinicalTrials.gov lists two phase II clinical trials that are still recruiting patients. One study is investigating the antineoplastic effect of the FGFR inhibitor pemigatinib as well as the therapeutic relevance of FGFR3 alterations in patients with recurrent low or intermediate risk NMIBC prior to second TUR-B (NCT03914794). The other study is examining the effect of erdafitinib versus either gemcitabine or mitomycin in patients with high-risk NMIBC and FGFR3 alterations with recurrence after BCG (NCT04172675).

The limitations of the current study include the retrospective nature as well as the relatively small cohort of 80 patients, which limits the reproducibility. In addition, no further subclassification with regard to the combined FGFR3 and CDKN2A expression was performed, as the small subgroups would not allow any meaningful conclusions. No immunohistochemistry was used, which is the most common method of marker quantification. There are several other biomarkers described with prognostic and/or predictive impact for NMIBC that were not included in this study.[36,55] Moreover, only mRNA expression levels and no mutational status was analyzed. However, a recent study in stage T1 NMIBC showed an association between FGFR3 mutations and higher expression of FGFR3 mRNA.[56] Finally, while we validated the association of clinicopathological features with FGFR3 and CDKN2A mRNA expression. However, we did not aim to replicate individual thresholds, although the interlab variation for mRNA analysis appeared to be reasonably low.[57] We suggest that future prospective clinical trials may determine valid thresholds for better implementation of mRNA quantification into daily clinical practice. Altogether, we could verify the association of FGFR3 and CDKN2A mRNA expression with long-term prognostic outcomes in NMIBC patients.

Conclusion

In conclusion, we were able to confirm the overall positive prognostic role of high FGFR3 mRNA expression in stage T1 NMIBC, although these tumors are associated with increased recurrence rates. A conclusive assignment to either basal or luminal subtypes is not possible in stage T1 NMIBC. High CDKN2A expression is associated with unfavorable outcomes. Additional studies are necessary to investigate the applicability and usefulness of anti-FGFR3-targeted therapies in the difficult-to-treat stage T1 NMIBC.

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