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Expression of programmed cell death protein 4 (PDCD4) and miR-21 in urothelial carcinoma

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ABSTRACT

Background: We investigated the role of the programmed cell death 4 (PDCD4) tumor suppressor gene in specimens of transitional cell carcinoma and of healthy individuals.
Methods: PDCD4 immunohistochemical expression was investigated in 294 cases in histologically proven transitional cell carcinoma in different tumorous stages (28 controls, 122 non-muscle invasive urothelial carcinoma, stages Tis-T1, 119 invasive transitional cell carcinoma stages T2–T4 and 25 metastases). MiR-21 expression, an important PDCD4 regulator, was assessed with real-time PCR analysis and showed inverse correlation to tissue PDCD4 expression.
Results: Nuclear and cytoplasmatic PDCD4 immunostaining decreased significantly with histopathological progression of the tumor ($p < 0001$). Controls showed strong nuclear and cytoplasmatic immunohistochemical staining. MiR-21 up regulation in tissue corresponded to PDCD4 suppression.
Conclusions: These data support a decisive role for PDCD4 down regulation in transitional cell carcinoma and confirm miR-21 as a negative regulator for PDCD4. Additionally, PDCD4 immunohistochemical staining turns out to be a possible diagnostic marker for transitional cell carcinoma.

1. Introduction

Programmed cell death 4 (PDCD4) is a tumor suppressor gene, which is down-regulated in many tumorous entities. PDCD4 binds to the eukaryotic translation initiation factors eIF4A and eIF4G and inhibits their function by preventing RNA binding (AP-1 transactivation) [1,2]. It is located on the chromosome 10q24. The translated protein consists of 469 aminoacids with two basic domains on the C- and N-terminus and two conserved alpha-helical MA-3 domains. The TGF-ß pathway may represent a major regulator of PDCD4 expression by inducing miR-21 (micro RNA 21). Asangani et al. showed the 3′-UTR region of the PDCD4 mRNA to be one target of the miR-21. High miR-21 concentrations led to a down-regulation of PDCD4 and an induction of invasion, intravasation and metastasis of tumor cells.[3] PDCD4 influences the level of different cellular translation and transcription pathways in different tumor entities: On the translational level, carbonic anhydrase II (CA II) is down-regulated in PDCD4 over-expressing cells (mock- and PDCD4 transfected HEK-293 (human embryonic kidney)) [4]. Furthermore, PDCD4 was found to interfere at the transcriptional level in the Sp1 (specificity protein 1)/Sp3 motifs of the promoter of the uPAR gene (urokinase receptor) via phosphorylation of the Sp transcription factors in colorectal cells [5]. The urokinase receptor uPAR promotes tumor cell invasion and metastasis and mediates degradation of extracellular matrix components. Thus, this pathway was not confirmed for breast cancer cells: There was no suppression of uPAR transcription by PDCD4 overexpression [6], reflecting a possible cell-type specific function of PDCD4 and its inconsistent involvment in carcinogenesis.

The expression of PDCD4 in urothelial carcinoma has not been reported to this date.

We demonstrate the stage-dependent suppression of PDCD4 and correlate these data with miR-21 expression in urothelial tumors.

Abbreviations: BC, bladder carcinoma; HI, healthy individuals; MIBC, muscle invasive bladder carcinoma; NMIBC, non-muscle invasive bladder carcinoma; NU, normal urothelial tissue.

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Table 1
Clinical pathological information of the cohort.

Variable	NMIBC	MIBC	NU
All *n*=	122	119	28
Age (mean; range)	66 (66; 51–81)	68.17 (70; 38–94)	66.5 (63.6; 51–81)
Sex (%)			
Male	99 (36.8)	86 (32)	19 (7.1)
Female	23 (8.6)	33 (12.3)	9 (3.3)
Smoking habits (%)			
Current	34 (16.3)	49 (23.6)	1 (0.5)
Former	9 (4.3)	6 (2.9)	0
Never	58 (27.9)	47 (22.6)	4 (1.9)
Tumor stage (%)			
Controls	n.a.	n.a.	28 (10.4)
pTa	67 (24.9)	0	n.a.
pTis	22 (8.2)	0	n.a.
pT1	33 (12.3)	0	n.a.
pT2	0	41 (15.2)	n.a.
pT3	0	50 (18.6)	n.a.
pT4	0	28 (10.4)	n.a.
N0	122 (50.6)	79 (32.8)	n.a.
N+	0	40 (16.6)	n.a.
M0	122 (51.3)	116 (48.7)	n.a.
M+	0	5 (2.1)	n.a.
Grade (%)			
G1	47 (19.5)	0	n.a.
G2	46 (19.1)	39 (16.2)	n.a.
G3	29 (12)	80 (33.2)	n.a.

Table 2
Clinical pathological information of the miR-21 cohort.

Variable	NMIBC	MIBC	NU
All *n*=	11	11	21
Age (mean; range)	70.27 (69; 52–84)	67.91 (70; 50–80)	47.81 (45; 36–83)
Sex (%)			
Male	9 (20.9)	11 (25.6)	7 (16.3)
Female	2 (4.7)	0	14 (32.4)
Tumor stage (%)			
pTa	7 (31.8)	0	n.a.
pT1	4 (18.2)	0	n.a.
pT2	0	2 (9.1)	n.a.
pT3	0	5 (22.7)	n.a.
pT4	0	4 (18.2)	n.a.
N0	11 (50)	5 (22.7)	n.a.
N+	0	6 (27.3)	n.a.
L0	11 (50)	4 (18.2)	n.a.
L1	0	7 (31.8)	n.a.
Grade (%)			
G1	4 (18.2)	0	n.a.
G2	7 (31.8)	3 (13.6)	n.a.
G3	0	8 (36.4)	n.a.

2. Material and methods

2.1. Patients

To study the expression of PDCD4 in BC and normal urothelial tissue, we used a tissue microarray described earlier [7]. The tissue microarray included tissue from non-muscle invasive (NMIBC; $n = 122$), muscle invasive urothelial carcinoma (MIBC; $n = 119$), metastatic BC ($n = 25$) and normal urothel ($n = 28$). See Table 1 for clinical pathological parameters.

To compare the expression of PDCD4 and miR-21, we analysed 11 patients with MIBC and 11 patients with NMIBC; corresponding normal urothelial tissue was also available from 3 patients with MIBC and 8 patients with NMIBC. See Table 2 for clinical pathological parameters.

2.2. Immunohistochemistry

All specimens were immediately fixed in 4% buffered formalin and embedded in paraffin wax. Serial histological sections 4–6 μm thick were obtained from each paraffin wax block and stained with H&E and Alcian-PAS. The original diagnosis was confirmed on the histological evidence in all cases by two independent observers. Tumor regions and control tissue were marked on H&E slides and three cores were punched out of the original paraffin block and transferred into a recipient block. These tissue micro arrays were used for further investigation.

Immunohistochemical staining was done automatically (DAKO TechMate™ 500, DAKO, Glostrup, Danmark) for PDCD4 (1:400, Anti-Pdcd4 (Rabbit) antibody, Rockland, Philadelphia, USA) according to the manufacturer's instructions (see Fig. 3). Appropriate positive and negative controls were run concurrently using rabbit igG-isotype. In cancer samples, the presence of positive stromal and inflammatory cells, as well as normal tissue served as an internal control. Two pathologists who were unaware of the patients' clinical history jointly scored PDCD4 expression. Consistent with Mudduluru et al. nuclear and cytoplasmic immunoreactions were considered [8]. Nuclear PDCD4 staining results were classified in four groups according to the percentage of positively stained nuclei: score 0, none; score 1, $\leqslant$30%; score 2, 30–70%; score 3, $\geqslant$70% (nuclear quantity). Cytoplasmic and nuclear PDCD4 expression was scored by staining intensity (score 0, none; score 1, weak; score 2, intermediate; score 3, strong). A total score was also calculated as the sum of the nuclear and cytoplasmic scores, and cases were divided into four groups, defined as: negative (total score 0), weak staining (total score 1 or 2), intermediate staining (total score 3 or 4), and strong staining (total score 5 or 6). We did not apply a quantity score for cytoplasma because positive staining was consistently 100%. All images were saved for further evaluation using a pathology scanner (Pannoramic MIDI Scanner, 3DHISTECH, Budapest, Hungary).

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated from formalin-fixed, paraffin embedded tissues. Five serial sections (20 μm) were cut for HE staining and at the end a 5 μm thick cut was taken for PDCD4 staining. Malignant and normal urothelial tissue was marked and subsequently microdissected using a scalpel and dissection needle. Tissue samples were deparaffinised with xylene and ethanol and finally air dried. Total RNA was extracted using the Recover All™ Total Nucleic Acid Isolation Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was determined photometrically (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA).

Reverse transcription was performed using 500 ng total RNA using the miScript Reverse Transcription kit according to the manufacturer's recommendations (miScript Reverse Transcription Kit, Qiagen Germany).

Quantitative real-time PCR was performed using the SYBR Green PCR Kit (miScript SYBR® Green PCR Kit, Qiagen, Germany) in triplicate on an ABIPrism 7900HT (Applied Biosystems, Foster City, CA, USA). Each 10 μl reaction contained 1x SYBR green master mix, 1 μl cDNA (12.5 ng/μl), 1 μl universal reverse primer and 1 μl gene-specific primer (primers purchased from Qiagen: miR21: MS00009079, RNU1A-1: MS00013986, RNU6B: MS00014000. RNU6B and RNU1A1 served as reference genes [9]. The PCR protocol was: Hot start activation for 15 min at 95 °C, followed by 40 cycles with denaturation (15 s, 94 °C), annealing (30 s, 55 °C) and extension (30seconds, 70 °C); specificity was confirmed by melting curve analyses. Every experiment included positive controls (TCCSUPP, RT4, T24 and RT112 cell-lines), as well as a DNA sample and

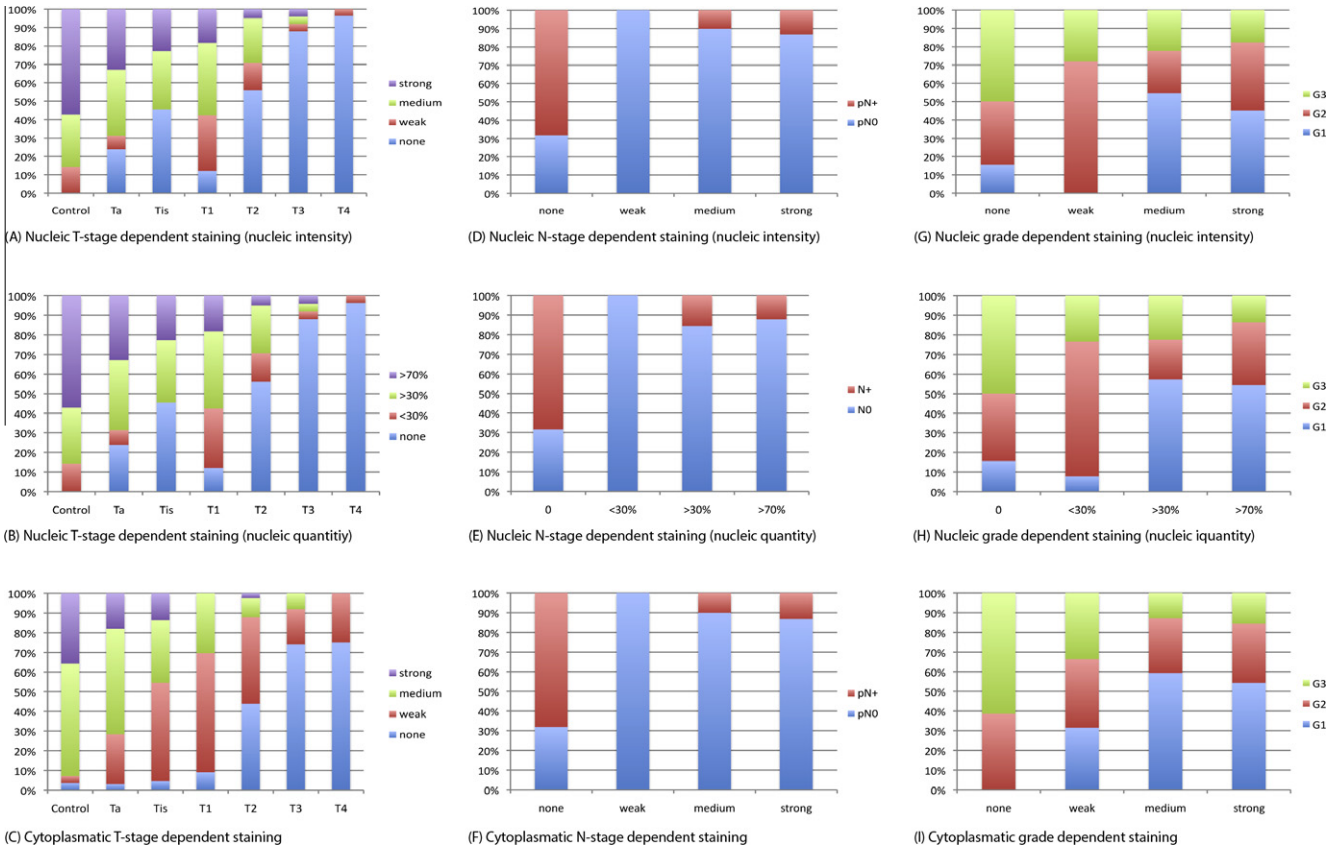

Fig. 1. PDCD4 expression levels depending on T- or N-staging and tumor grade. Nuclear (A, D and G) and cytoplasmic (C, F and I) intensity were scored in four groups according to the IHC staining intensity. Nuclear quantity (B, E and H) being classified according to the percentage of positively stained nuclei in four groups.

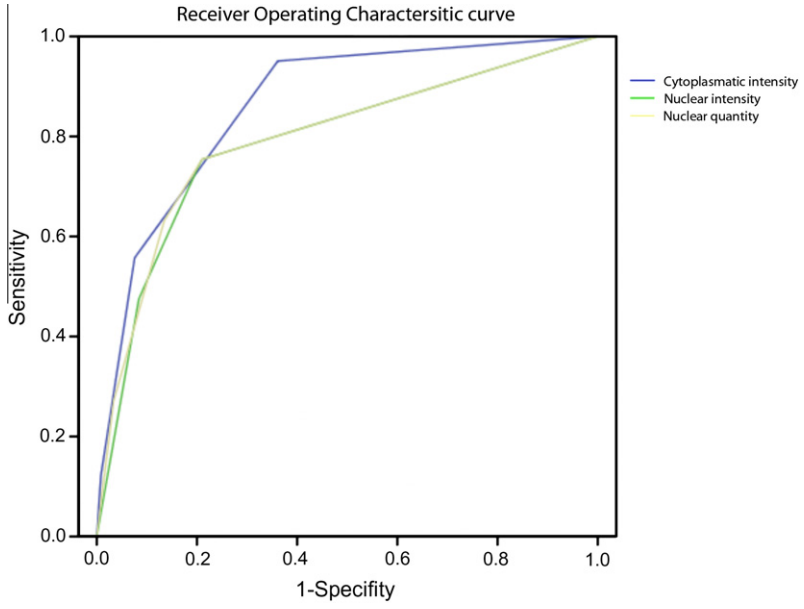

Fig. 2. ROC curve. The analysis shows a high specifity (75.4–95.1%) and sensitivity (63.9–79.0%) of the IHC stainings for NMIBC/MIBC discrimination.

water blanks as negative controls. Relative expression levels of miR-21 were calculated using the DataAssist Software (Applied Biosystems) using the ΔΔ-Ct-formula.

2.4. Statistical analysis

Clinical-pathological parameters were correlated with PDCD4 expression using the chi-square-test. The Cox proportional hazard regression analysis was used to correlate the period of progression-free survival and cancer-specific survival following surgery with PDCD4 levels. Receiver Operating Characteristics (ROC) curves were calculated to determine the specificity and sensitivity of PDCD4 expression in MIBC and NMIBC. The expression of miR-21 in PDCD4 negative and positive tissue samples was compared using the Mann–Whitney-*U*-test. All statistical analyses were done using Statistical Package for the Social Sciences 19 (SPSS, ©IBM

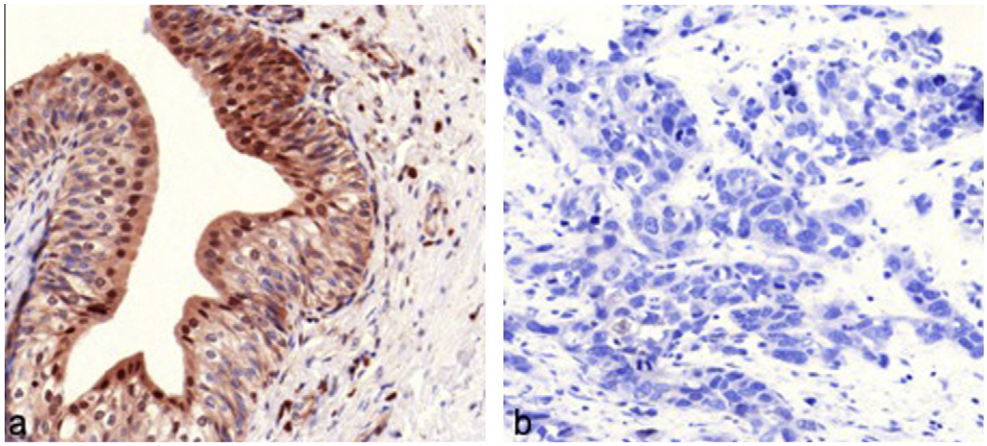

Fig. 3. Programmed cell death 4 (Pdcd4) immunostaining of normal urothelial tissues and T4 invasive urothelial carcinoma (20×). (a) Normal urothel with strong nuclear and cytoplasmatic staining, especially in top layer. (b) T4 urothelial carcinoma. Nuclear and cytoplasmatic staining is lost completely compared to normal tissue. Each staining was performed with negative control.

Table 3
p-Values (chi-square test) of the PDCD4 IHC correlations.

p-Values	Nuclear intensity	Nuclear quantity	Cytoplamatic
NMIBC			
T-Stage	0.023	0.002	0.004
Grading	0.131	0.004	0.069
N0 /N+	n.a.	n.a.	n.a.
Smoker	0.268	0.171	0.276
Sex	0.279	0.105	0.578
MIBC			
T-Stage	0.001	0.001	0.032
Grading	0.448	0.802	0.377
N0 /N+	0.068	0.054	0.068
Smoker	0.66	0.845	0.141
Sex	0.441	0.232	0.927
M0/M1	0.691	0.165	0.384
All patients			
BCA/Met/Control	<0.001	<0.001	<0.001
NMIBC vs. MIBC	<0.001	<0.001	<0.001
Sex	0.405	0.201	0.364
Smoker	0.094	0.137	0.267
T-Stage	<0.001	<0.001	<0.001
Grading	<0.001	<0.001	<0.001
M0/M1	0.565	0.451	0.144
N-Stage	<0.001	<0.001	<0.001
N0/N+	<0.001	<0.001	<0.001
Stage & Grade			
Ta vs. Tis	0.072	0.178	0.162
Tis vs. T1	0.015	0.005	0.161
Ta vs. T1	0.123	0.012	0.001
T2 vs. T3	0.006	0.004	0.021
T2 vs. T4	0.003	0.003	0.048
T3 vs. T4	0.405	0.496	0.236
Met vs. T2	0.533	0.220	0.507
Met vs. T3	0.149	0.086	0.210
Met vs. T4	0.036	0.042	0.352
G1 vs. G2	0.005	<0.001	<0.001
G1 vs. G3	<0.001	<0.001	<0.001
G2 vs. G3	0.012	0.003	0.017

Table 4
ROC curve analysis.

	Sensitivity	Specifity	AUC	95% CI
Cytoplasmatic intensity	95.1%	63.9%	0.862	0.815–0.909
Nucleic intensity	75.4%	79.0%	0.790	0.732–0.849
Nucleic frequency	75.4%	79.0%	0.795	0.737–0.853

Corporation, Somer NY, USA). Statistical significance was concluded at $p < 0.05$.

3. Results

3.1. PDCD4 levels are different in normal urothelial and bladder cancer tissue

We first investigated cytoplasmic and nucleic PDCD4 expression in NMIBC, MIBC, Mets and NU tissue. Cytoplasmic and nucleic PDCD4 expression was highest in NU, and decreased from NMIBC to MIBC and METS. See Fig. 1 and Table 3 for details.

3.2. PDCD4 expression levels show high sensitivity and specificity for MIBC and NMIBC discrimination

We calculated Receiver Operating Characteristic (ROC) curves to investigate sensitivity and specificity of PDCD4 expression levels in MIBC and NMIBC: PDCD4 staining allowed sensitive and specific discrimination (see Table 3 and Fig. 2); the AUC (area under the curve) was >0.79 for nucleic/cytoplasmic intensity respectively nucleic frequency (see Table 4).

3.3. PDCD4 expression is associated with advanced pathological stage

PDCD4 expression seems to be of prognostic relevance in BC patients because global levels of these modifications were correlated with staging and grading. For example, cytoplasmatic expression levels were increased in NMIBC patients with advanced pT-stage (pTa vs. pT1, $p = 0.001$; pTa vs. pTis, $p = 0.162$) and less differentiated BC (G1 vs. G2, $p < 0.001$; G1 vs. G3, $p < 0.001$; G2 vs. G3, $p = 0.019$). Consistently, in patients with MIBC the pT-stage was negatively correlated with nuclear PDCD4-expression (pT2 vs. pT3, $p = 0.006$; pT2 vs. pT4, $p = 0.003$; pT3 vs. pT4 $p = 0.405$) levels. T-stage, tumor grading and nodal status showed a high correlation

Table 5
IHC staining results from metastasy samples.

	NI (%)	NQ (%)	C (%)
None	18 (72)	18 (72)	15 (60)
Medium	3 (12)	5 (20)	9 (36)
Strong	4 (16)	2 (8)	1 (4)

NI: Nuclear intensity, NQ: Nuclear quantity (<30%/>30%/>70%), C: Cytoplasmatic.

Table 6
COX-regression analysis of the PDCD4 staining results for NMIBC and MIBC.

	CSS univariate analysis			Recurrence-free survival univariate analysis		
	p-Value	Mean	95%-CI	*p*-Value	Mean	95%-CI
NMIBC						
pT-Stage	0.888	1.712	0.36–3.248	0.793	1.654	0.737–1.262
Grading	0.62	1.865	0.379–5.089	0.419	1.808	0.824–1.592
PDCD4 nuclear intensity	0.144	1.919	0.256–1.221	0.46	1.962	0.888–1.3
PDCD4 nuclear quantity	0.267	1.613	0.247–1.473	0.778	1.663	0.845–1.253
PDCD4 cytoplamatic	0.8	1.604	0.061–1.173	0.5	1.644	0.647–1.237
MIBC						
pT-Stage	<0.001	4.891	1.438–3.606	0.015	4.865	1.083–2.120
Grading	0.44	2.672	1.023–5.031	0.358	2.64	0.477–1.307
M0/M1	<0.001	0.043	4.537–46.824	0.549	0.011	0.251–13.430
Nodal status	0.217	0.336	0.775–3.070	0.426	0.315	0.463–1.384
PDCD4 nuclear intensity	0.535	0.496	0.804–1.519	0.821	0.438	0.747–1.261
PDCD4 nuclear quantity	0.077	0.378	0.963–2.092	0.676	0.326	0.756–1.538
PDCD4 cytoplamatic	0.767	0.445	0.571–1.512	0.209	0.416	0.515–1.157

level ($p < 0.001$). However, no correlation between PDCD4 expression and gender, smoking habits or M-stage was revealed. Although expression levels in cytoplasmatic and nuclear intensity were decreasing with tumor grading progression, differences did not reach significance in NMIBC. A detailed summary is provided in Table 3.

Analysis of the metastases showed a concomitant under-expression of IHC-staining intensity in all cellular compartments. PDCD4 expression levels in metastatic tissue correlate to the primary tumor expression levels. A detailed summary is provided in Table 5.

3.4. PDCD4 expression is not correlated with patients' outcome

Furthermore, we performed Cox proportional hazard models to analyze the prognostic value of PDCD4 levels on patients' outcome. Follow up information was available for 111 patients with NMIBC and 119 patients with MIBC. Recurrence was observed in 73 NMIBC and 62 MIBC; 34 patients with MIBC and 7 with NMIBC died from BCA. The NMIBC group showed no significant results, whereas in the MIBC group the analysis showed a highly significant correlation to tumor stage (T-stage) and to the metastatic status of the patients (M-stage). Detailed information is provided in Table 6.

3.5. PDCD4 and miR-21 expression in neoplastic tissue

MiR-21 regulates PDCD4 via binding to its 3'UTR region. Therefore, we determined whether miR-21 expression correlated to PDCD4-expression in BC: MiR-21-expression was increased in neoplastic tissue consistently to the tumor stage (NMIBC/MIBC) and correlated inversely to PDCD4 expression in tissue ($p = 0.018$). Concordantly to these results, controls showed under-expression of miR-21 and strong IHC staining for PDCD4 (Table 2, Pearson's, $p < 0.05$).

4. Discussion

In the present study, we demonstrate that nuclear and cytoplasmatic PDCD4 is downregulated in BC, and that downregulation is correlated with advanced stage and grade of BC. The results of Denzinger et al. showed the clinical importance of a discriminative marker for MIBC and NMIBC in pT1 G3 BCs [10]. In this study, recognised risk factors in resected BC turned out to be of no predictive value for cancer-related death, only carcinoma in situ was related to a lower CSS. After cystectomy, upgrading was found in 30% of cases and no risk factor was related to upstaging. A high specifity and sensitivity of PDCD4 expression as shown in the ROC curve analysis suggest PDCD4 as a potential diagnostic marker. Further investigation regarding the discriminative power of PDCD4 staining results in a prospective study is mandatory.

PDCD4 seems to play an important role in various human malignancies: Loss of expression was reported in several types of human cancer cell lines [11], lung cancer [12], colorectal carcinoma [8], hepatocellular carcinoma [13], breast cancer [14], tongue cancer [15] and glioma [16]. In colon carcinoma cell lines, loss of PDCD4 promoted invasion [17]. Göke et al. also demonstrated that PDCD4 induced the expression of the (CDK)1/cdc2 cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ [18]. In summary, these findings indicate that PDCD4 acts as tumor suppressor gene. Targeting PDCD4 could be a future therapeutic option: In a lung cancer mice model, aerosol delivery of a PDCD4-complex faciliated apoptosis and inhibited pathways for cell proliferation and tumor angiogenesis [19].

PDCD4 expression was strongly associated with advanced stage and grade of BC. This finding indicates a prognostic role of PDCD4 in BC. However, we did not observe a correlation of PDCD4 expression and progression-free- or cancer-specific survival. This may be due to the limited amount of PDCD4 positive MIBC samples, which made a robust survival analysis problematic. However, loss of PDCD4 was a predictor of cancer-specific survival in patients with ovarian and colon cancer [8,20]. Earlier studies indicated that PDCD4 expression is controlled by microRNAs: Davis et al. demonstrated that TGF-beta induced the expression of mature miR-21 in smooth muscle cells; miR-21 in turn suppressed the PDCD4 levels. A conserved target site for miR-21 is located in the 3'UTR of the PDCD4 mRNA. Thus, transfection of miR-21 lead to a decrease of PDCD4. Thereby, miR-21 expression lead to invasion, intravasation and metastasis in a chicken-embryo-metastasis assay and on glioblastoma cells [3,21]. Our finding of increased miR-21 levels in tissue without PDCD4 expression is consistent with earlier studies in cell lines and confirms the role of miR-21 in the regulation of PDCD4 in primary tumor cells.

Our study demonstrates that PDCD4 is down-regulated in BC and is associated with advanced stage and grade. Downregulation of PDCD4 is induced by miR-21.

5. Disclosure statement

The authors declare that they have no competing interests.
The authors declare that they have no financial interests.

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