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Increased expression of DCLK1, a novel putative CSC maker, is associated with tumor aggressiveness and worse disease-specific survival in patients with bladder carcinomas

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ABSTRACT

Doublecortin-like kinase 1 (DCLK1) has been characterized as a novel potential cancer stem cell (CSC) marker in several types of cancer. It is considered as one of the most specific markers for distinguishing colorectal CSCs from normal stem cells. Yet, there are limited reports on the role of DCLK1 as a putative CSC marker in bladder cancer. Using immunohistochemistry, DCLK1 expression was examined in a well-defined tissue microarray series of 472 bladder cancer tissues. The association between DCLK1 protein expression and clinicopathological features, as well as survival outcomes, was assessed. Our findings showed strong, moderate, and weak DCLK1 expression in 123 (26.1%), 230 (48.7%), and 119 (25.2%) of the bladder cancer specimens, respectively. Higher expression of DCLK1 was significantly associated with increase in histological grade ($P \leq .001$), pT stage ($P = .014$), lamina propria ($P = .006$), and lamina propria/muscularis (L/M) involvement ($P = .014$). On multivariate analysis, pT stage ($P < .001$), histological grade ($P = .021$), and lamina propria involvement ($P = .001$) were independent prognostic factors in DCLK1 expression. Moreover, the expression of DCLK1 was found to be an independent marker of poor prognosis for disease- specific survival (DSS) ($P = .048$) in bladder carcinomas. Our observations showed that DCLK1 expression was associated with more aggressive tumor behavior, more advanced disease, and poorer DSS in patients with bladder carcinomas. However, any potential clinical applications of DCLK1 as a novel target molecule in bladder cancer patients would require further investigations.

1. Introduction

Bladder cancer is the most common cancer of the urogenital tract and the ninth death leading cause of cancer worldwide (Burger et al., 2013). About 90% of bladder cancers are transitional cell carcinoma (TCC), the remainder include squamous cell carcinoma (SCC), adenocarcinoma, small cell carcinoma (SCLC), and metastatic tumors (Ferlay et al., 2015b, 2013). TCCs are consisted of two subtypes: low-grade/surface form that often are papillary and multi focal, and high-grade approximately half of which are metastatic at the time of diagnosis (Ferlay et al., 2015a, 2013). However, tumor recurrence is frequently occurring in high grade tumors. The treatment of bladder cancers in low grade and early TNM stage cases predominantly includes surgery, chemotherapy, and radiotherapy (Zhang et al., 2017). Combining these traditional methods with new targeted- therapy strategies could open new doors for the prognosis prediction of bladder cancer. Hence, the identification of the biomarkers is considered to be an essential step for improving targeted molecular therapies and avoiding the recurrence of tumors (Becker et al., 2008; Ho et al., 2012; Kim et al., 2013; Zhang et al., 2016).

Cancer stem cells (CSCs) within the bulk of a tumor, through characteristics such as self- renewal, tumor-initiating, and drug-

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resistance behavior, are believed to lead to progression, migration, and metastasis (Dou and Gu, 2010; Tirino et al., 2013; Zhang et al., 2016). The identification and isolation of CSC populations have implicated various surface markers in different hematopoietic and solid tumors; including breast, lung, prostate, and intestinal tumors (Gheytanchi et al., 2014; Kalantari et al., 2017c; Liu et al., 2016; Nakanishi et al., 2013; Roudi et al., 2015). Within bladder cancer tumors, CD44, CD133, ALDH1, and OCT4 have previously been reported as CSC markers (Amini et al., 2014; Chan et al., 2009; Jaggupilli and Elkord, 2012; Keymoosi et al., 2014; Sabet et al., 2014; Sedaghat et al., 2017; Su et al., 2010).

Doublecortin-like kinase 1 (DCLK1) is a microtubule- associated protein and a specific gastrointestinal CSC marker, which has been widely studied within different solid tumors; including pancreatic, colorectal, intestinal, prostate, and hepatocellular carcinomas (Bailey et al., 2014; Fan et al., 2017; Jiang et al., 2018; Li and Bellows, 2013; May et al., 2008; May et al., 2010; Nakanishi et al., 2013; Sureban et al., 2015). The key role of DCLK1 in tumorigenesis, proliferative potential, angiogenesis, epithelial–mesenchymal transition (EMT), invasion, apoptosis, and metastasis have previously been confirmed through in vivo and in vitro studies in colorectal, pancreatic, and renal tumors (Bailey et al., 2014; Chandrakesan et al., 2015; Ikezono et al., 2017; Li and Bellows, 2013; May et al., 2008; May et al., 2010; Westphalen et al., 2016; Weygant et al., 2015). It has been suggested that various DCLK1 isoforms could play different, but specific biological functions in the tumorigenesis and aggressive behavior of tumor cells, however many confusions remain on the mechanistic details (O'Connell et al., 2015). Through using a home-made mono-specific DCLK1-S antibody, Sarkar et al. showed the higher expression of DCLK1-S in 82 colorectal cancer tumors, they found a significant negative correlation between the expression of DCLK1-S and overall survival using qRT-PCR; indicating that the antibody may act as a prognostic marker in high-risk colorectal cancer patients at the time of the colonoscopy (O'Connell et al., 2015; Sarkar et al., 2017). Further evidence from in vivo studies indicate higher DCLK1-L expression, supporting the notion that it plays an important role in tumorigenesis at the early stages of colorectal cancer and pancreatic cancer (Bailey et al., 2014; Westphalen et al., 2014; Westphalen et al., 2016). Our previous findings have demonstrated various expression patterns for DCLK1 in colorectal and gastric carcinomas (Kalantari et al., 2017b; Mirzaei et al., 2015). In colorectal tissues, we found that DCLK1 expression has a significant direct correlation with TNM stage and grade, while in gastric carcinoma tissues, a higher expression of DCLK1 was found in the intestinal subtype and well-differentiated specimens (Kalantari et al., 2017b; Mirzaei et al., 2015). A higher expression of DCLK1 has been reported in tumoral tissues compared to normal colorectal, renal, and plural samples (Mirzaei et al., 2015; Mohammadi et al., 2018; Wang et al., 2017; Weygant et al., 2015). While the role of DCLK1 in the stemness of colorectal and pancreatic cancers has been well studied, there have been less studies on the expression pattern and CSC function in other tissues such as bladder carcinomas (Chandrakesan et al., 2015; Ikezono et al., 2017; Mirzaei et al., 2015; Qu et al., 2015; Westphalen et al., 2016). The only study, which was recently (2017) performed on a series 118 bladder cancer tissue samples, showed an increased level of DCLK1 protein expression in aggressive TNM stage and metastatic specimens (Zhang et al., 2017).

As mentioned, the oncogenic role of DCLK1 as a potential CSC marker has been well-studied in a broad range of malignant tumors, whereas the clinical significance of this marker in bladder carcinoma has received far less attention. Here, we investigated the expression pattern of DCLK1 through immunohistochemical staining, on a tissue microarray (TMA) series including 472 TCC and 29 normal samples. Furthermore, the association of DCLK1 expression with clinicopathological features and survival outcomes in bladder cancer patients was evaluated.

2. Materials and methods

2.1. Tissue collection and TMA construction

A total of 472 TCC samples, including transurethral resection of bladder tumor (TURB) and 16 matched adjacent non-cancerous normal tissues were collected between 2008 and 2011 from bladder cancer patients at Hasheminejad Hospital, a major Urology-Nephrology referral hospital in Tehran, Iran. None of the bladder cancer patients in this study had received neoadjuvant treatment before surgery.

The clinicopathological characteristics, including age, gender, tumor size (maximum diameter), histological grade, pT stage, lamina propria involvement, muscularis propria invasion, distant metastasis, and recurrence were recorded for each sample. The cutoff size and the pT stage of tumors were determined according to the American joint committee on cancer/international union against cancer (AJCC/UICC) and pTNM staging system, respectively (Cheng et al., 2009; Grignon, 2009; Sobin and Compton, 2010). Information about patients' outcome; including the time between TURB and cancer-related death, or last follow-up (if death did not occur), and TURB and last follow-up (if the patient showed no evidence of disease), recurrence, or metastasis of bladder carcinoma, or cancer-related death, were also recorded. This study was approved by the Iran University of Medical Sciences Research Ethics Committee.

The Hamatoxylin and Eosin (H&E) slides were used to select and point three representative areas of the tumor by a pathologist (Kalantari et al., 2017b; Korourian et al., 2017). The TMA recipient blocks were constructed by a TMA instrument (Minicore; ALPHELYS, Plaisir, France), as described previously (Kalantari et al., 2017a; Kalantari et al., 2017c; Keymoosi et al., 2014; Mohsenzadegan et al., 2013; Roudi et al., 2014; Sabet et al., 2014; Sedaghat et al., 2017). In TMA-based studies, each core would denote the staining pattern of the whole tissue with 90% accuracy, while using two cores or more could increase this accuracy to 95–99%, respectively (Camp et al., 2000). In this study, to overcome the heterogeneity of antigen expression and increase the accuracy and validity of analysis, three cores were evaluated from each tissue sample.

2.2. Immunohistochemistry

Standard chain polymer-conjugated antibody (Envision) was used to stain the formalin-fixed, paraffin-embedded sections of the TMA constructed slides, as described previously (Kalantari et al., 2017b; Korourian et al., 2017; Mehrazma et al., 2013; Saeednejad Zanjani et al., 2019). The TMA sections were dewaxed and rehydrated by xylene and graded ethanol treatment. The samples were incubated overnight with anti-DCLK1 (1:1000 dilution, ab31704; Abcam, UK), at 4 °C, followed by antigen retrieval in citrate buffer (pH = 6.0), and then autoclaved for 11 min. The sections were then treated with the secondary antibody- standard EnVision-HRP kit (DAKO, Denmark) for one hour, at room temperature (RT). This was followed by visualization with 3; 3′-diaminobenzidine (DAB, Dako) substrate as a chromogen for 2 min at RT, the sections were counterstained with hematoxylin for 15 min, dehydrated, and finally mounted. Human bladder cancer tissue was selected as the positive control, and the replacement of the primary antibody with Tris Buffer Saline (TBS) wash buffer was used as the negative control (Gao et al., 2015).

2.3. Scoring system of TMA slides

The TMA tissue sections were scored semi-quantitatively, separately, by two pathologists who were blinded to the clinicopathologic parameters associated with each sample (Mehrazma et al., 2013; Mohsenzadegan et al., 2013; Sabet et al., 2014; Taeb et al., 2014). Immunohistochemical staining of slides was carried out, as describe previously (Nagel et al., 2004). In order to assess the DCLK1 expression,

the final scoring evaluation was carried out with reinvestigation of the overall distribution of the tumor cells at 10× magnification. Positive cells were then assessed, semi-quantitatively, at higher magnifications (20× or 40×). Final scores were given following an agreement between scorers. The intensity of immunostaining was categorized into four groups: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage of positive cells were valued semi-quantitatively, and scored as 0–100%. The *H*-score was obtained by multiplying the intensity (0–3) and percentage scores (0–100%), and generated scores of 1–100, 100–200, and 200–300 (McCarty et al., 1985; McCarty et al., 1986). The median *H*-score (=100) was chosen as the cutoff point. The specimens with *H*-score ≤ 100 were considered to be low DCLK1 expressing tissues, and the specimens with *H*-score ≥ 100 were considered to be DCLK1 tissues (Kalantari et al., 2017b; Roudi et al., 2015).

2.4. Statistical analysis

The SPSS software version 22 (SPSS, Chicago, IL, USA) was used to analyze the results. We reported the categorical data by N (%), valid percentage and quantitative data as follows, mean (SD) and median (Q1, Q3). Kruskal-Wallis and Mann-Whitney *U* tests were done for pairwise comparison between groups. Moreover, the association between DCLK1 expression and clinicopathological features was carried out by Pearson's Chi-square test. Disease-specific survival (DSS) was measured from the date of TURB, to the date of death caused due to bladder cancer. Progression-free survival (PFS) was defined as the interval between primary surgery and the last follow-up visit without disease, evidence of recurrence or progression. The DSS and PFS were estimated using Kaplan–Meier method with 95% confidence intervals (CI) and compared across the groups using the log-rank test. The Cox proportional hazards regression model was applied to determine which variables influenced survival. Variables that significantly influenced survival in univariate analysis were included in multivariable analysis. A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Study population

The sample population included a total of 472 TCC patients. The median age of the patients was 65 years, (ranging from 22 to 95); 245 (51.9%) patients were younger than 65 years old and 227 (48.1%) were over 65. The study population consisted of 389 (82.4%) male and 83 (17.6%) female patients, with a male/female ratio of 4.6. Tumor size (at the largest diameter) ranged from 1 to 13 cm, and tumors were classified into two groups, based on mean tumor size (2.5 cm). The patient's clinicopathological features are summarized in Table 1.

3.2. Expression of DCLK1 in bladder carcinoma

The immunohistochemical analysis for DCLK1 showed cytoplasmic expression patterns in both bladder tumor cells and adjacent normal tissues. However, the overexpression of DCLK1 was more distinct in tumor cells, compared to normal tissues - which only showed faint staining. No significant staining of DCLK1 expression was observed in the stroma. The comparison of overall staining based on the median *H*-score (= 100) as the cut off, showed lower DCLK1 expression in 238 (50.4%) samples, versus higher DCLK1 expression in the remaining 234 (49.6%) samples. Moreover, DCLK1 expression was observed with a variety of intensities among the samples (Fig. 1). Of 472 samples, 119 (25.2%), 230 (48.7%) and 123 (26.1%) showed weak, moderate, and strong immunoreactivity, respectively. These findings are summarized in Table 2.

Table 1
Patients and pathological characteristic of bladder carcinoma tissues.

Patients and tumor characteristics	Total samples N (%)
No. tumor samples	472
Median age	65
Years (Range)	73
65≤	245 (51.9)
65 >	227 (48.1)
Gender	
Male	389 (82.4)
Female	83 (17.6)
Tumor size (cm)	
2.5 ≤ Mean	288 (61.0)
2.5 > Mean	184 (39.0)
Histological grade	
Low	264 (55.9)
High	208 (44.1)
pT stage	
pTa	262 (55.5)
pT1	157 (33.3)
pT2	53 (11.2)
pT3	0 (0.0)
pT4	0 (0.0)
Lamina propria involvement	
Involved	208 (44.1)
None	264 (55.9)
Muscularis invasion	
Involved	53 (11.2)
None	419 (88.8)
lamina propria/muscularis involvement (L/M)	
L−/M-	262 (55.5)
L+/M-	157 (33.3)
L+/M+	53 (11.2)
Recurrence	
Present	112 (23.7)
Absent	360 (76.3)
Distant metastasis	
Present	62 (13.1)
Absent	410 (86.9)

N (%) shows number of cases and their percentage in brackets.

3.3. The association of DCLK1 expression with clinicopathological parameters

We found a significant difference in DCLK1 expression between samples with different histological grade ($P < .001$), pT stage (intensity $P < .001$, H-score $P = .014$), lamina propria involvement (Intensity $P < .001$, H-score $P = .006$), and L/M involvement (Intensity $P < .001$, H-score $P = .014$) in terms of both intensity of staining and *H*-score. In terms of *H*-score; lower DCLK1 expression was observed in 152 (57.6%) of low grade and 86 (41.3%) of high grade tumors. Conversely, higher DCLK1 expression was found in 112 (42.4%) low grade and 122 (58.7%) high grade tumors. From 262 tumors with pTa stage; the higher DCLK1 expression was seen in 115 (43.9%) of pTa stage, 92 (58.6%) of pT1 stage, and 27 (50.9%) of pT2 stage specimens. Of 208 cases with positive lamina propria involvement; 90 (43.3%) samples had lower and 118 (56.7%) cases showed higher DCLK1 expression. From 262 cases with L−/M- involvement; the higher expression of DCLK1 was seen in 115 (43.9%) of L−/M-, 92 (58.6%) of L+/M-, and 27 (50.9) of L+/M+ specimens. Furthermore, from an intensity of staining standpoint, a direct significant association was found between DCLK1 expression with age ($P = .009$), tumor size ($P = .018$), muscularis invasion ($P < .001$), and distant metastasis ($P = .042$). There was no significant association between DCLK1 expression and recurrence, neither based on intensity of staining, nor *H*-score. The observations have been summarized in Table 3. The non-parametric Kruskal–Wallis and Mann–Whitney *U* tests were used to compare differences between median DCLK1 expressions among grades and lamina propria involvement and non-involvement. Results of the Kruskal–Wallis test indicated a statistically significant difference

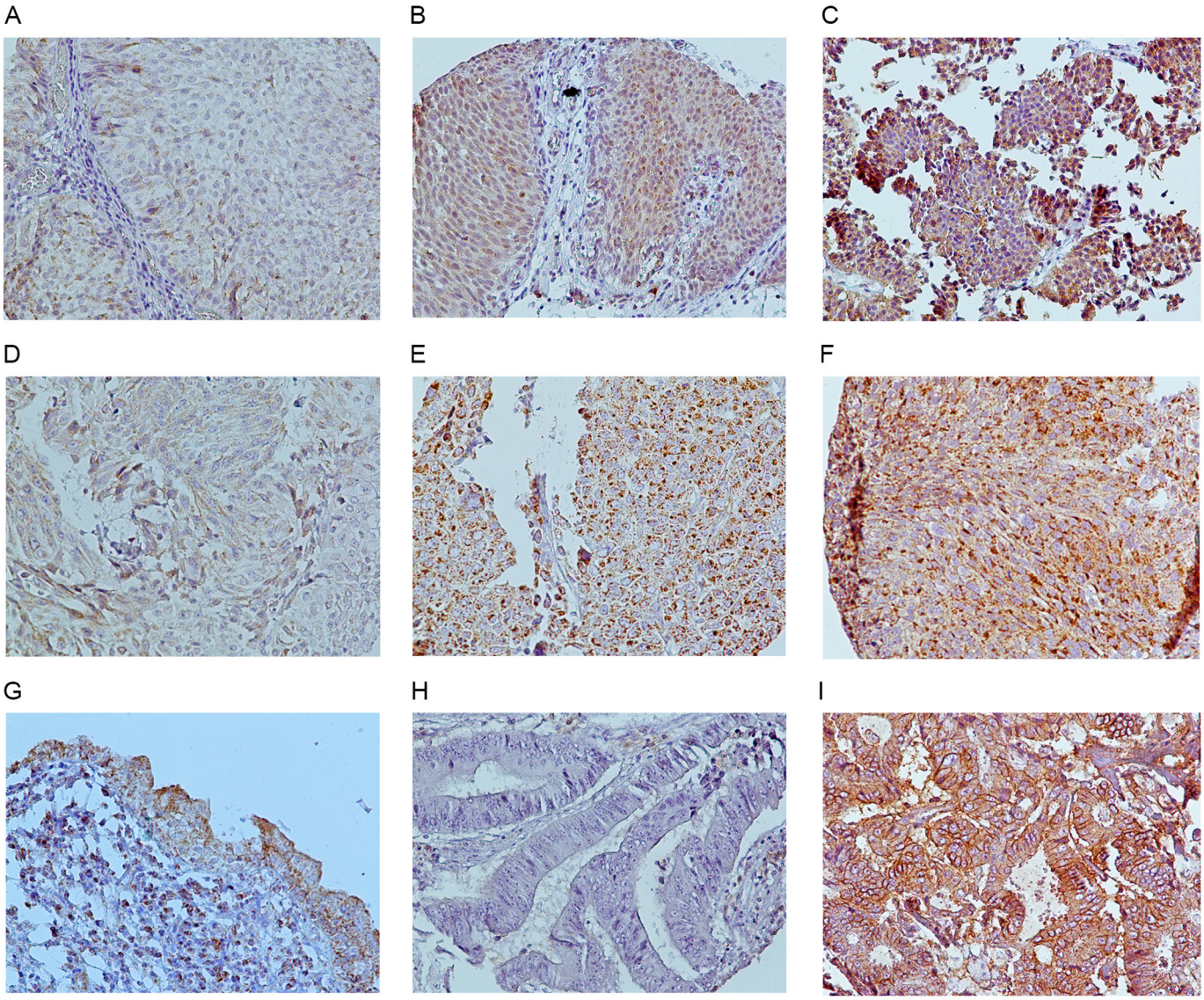

Fig. 1. Immunohistochemical staining of DCLK1 in bladder cancer (low and high grade) tissues, adjacent normal tissue, and colorectal cancer controls. (A) weak, (B) moderate, and (C) strong immunoreactivity of DCLK1 in low grade bladder carcinomas. (D) weak, (E) moderate, and (F) strong immunoreactivity of DCLK1 in high grade bladder carcinomas. (G) DCLK1 expression in adjacent normal tissue. (H) DCLK1 expression in human colorectal cancer as a negative control, and (I) DCLK1 expression in colorectal cancer as a positive control. (All images were taken at 200× magnification).

Table 2
DCLK1 expression (Intensity of staining and H-score) in bladder carcinoma.

Scoring system	Bladder	
	Carcinoma	Normal
	N (%)	N (%)
Intensity of staining		
Negative (0)	0 (0.0)	0 (0.0)
Weak (+1)	119 (25.2)	5 (17.2)
Moderate (+2)	230 (48.7)	21 (72.4)
Strong (+3)	123 (26.1)	3 (10.3)
H-score		
Low (≤100)	238 (50.4)	29 (100.0)
High (> 100)	234 (49.6)	0 (0.0)
Total	472	29

H-score indicates histological score.
N (%) shows number of cases and their percentage in brackets.

between the level of DCLK1 expression in various grades ($P < .001$) and DCLK1 expression in lamina propria involvement and non-involvement ($P = .016$). Therefore, Mann–Whitney U test was carried out and results showed that highly significant difference in the median of DCLK1 expression between low grade and high grade ($P < .001$) and lamina propria involvement and non-involvement ($P = .016$) (Fig. 2A, B). The bold line is meant to represent the median DCLK1 expression levels.

3.4. The prognostic value of DCLK1 expression

Of the 472 bladder samples that were included in this study, 358 (75.8%) were from patients that had no history of recurrence, metastasis, or cancer-related death, while 114 (24.2%) were positive for these events. Metastasis and recurrence occurred in 62 (13.1%) and 112 (23.7%) patients whereas 410 (86.9%) and 360 (76.3%) patients were negative, respectively. During the follow-up period, cancer-related death and the other cause of deaths were documented in 85 (18.0%) and 30 patients (6.4%), respectively. The mean and median follow-up

Table 3
The association between DCKL1 expression and clinicopathological parameters in bladder carcinoma (Intensity of staining and H-score) (*P value*; Pearson's χ^2 test).

Patients and tumor characteristics	Total no. cases	Intensity of staining N (%)				P- value	H-score (cut off = 100) N (%)		P- value
		0 (Negative)	1+ (Weak)	2+ (Moderate)	3+ (Strong)		Low (≤100)	High (> 100)	
Bladder carcinoma tissues	472	0 (0.0)	119 (25.2)	230 (48.7)	123 (26.1)	**0.009**	238 (50.4)	234 (49.6)	0.054
Age (years)									
65≤	245 (51.9)	0 (0.0)	76 (31.0)	113 (46.1)	56 (22.9)		134 (54.7)	111 (45.3)	
65 >	227 (48.1)	0 (0.0)	43 (18.9)	117 (51.5)	67 (29.5)		104 (45.8)	123 (54.2)	
Gender									
Male	389 (82.4)	0 (0.0)	98 (25.2)	182 (46.8)	109 (28.0)	0.083	195 (50.1)	194 (49.9)	0.781
Female	83 (17.6)	0 (0.0)	21 (25.3)	48 (57.8)	14 (16.9)		43 (51.8)	40 (48.2)	
Tumor size (cm)									
2.5≤	288 (61.0)	0 (0.0)	83 (28.8)	141 (49.0)	64 (22.2)	**0.018**	150 (52.1)	138 (47.9)	0.367
2.5 >	184 (39.0)	0 (0.0)	36 (19.6)	89 (48.4)	59 (32.1)		88 (47.8)	96 (52.2)	
Histological grade									
Low	264 (55.9)	0 (0.0)	106 (40.2)	134 (50.8)	24 (9.1)	< **0.001**	152 (57.6)	112 (42.4)	< **0.001**
High	208 (44.1)	0 (0.0)	13 (6.3)	96 (46.2)	99 (47.6)		86 (41.3)	122 (58.7)	
pT stage									
pTa	262 (55.5)	0 (0.0)	96 (36.6)	132 (50.4)	34 (13.0)	< **0.001**	147 (56.1)	115 (43.9)	**0.014**
pT1	157 (33.3)	0 (0.0)	20 (12.7)	77 (49.0)	60 (38.3)		65 (41.4)	92 (58.6)	
pT2	53 (11.2)	0 (0.0)	3 (5.7)	21 (39.6)	29 (54.7)		26 (49.1)	27 (50.9)	
Lamina propria involvement									
Involved	208 (44.1)	0 (0.0)	23 (11.1)	97 (46.6)	88 (42.3)	< **0.001**	90 (43.3)	118 (56.7)	**0.006**
None	264 (55.9)	0 (0.0)	96 (36.4)	133 (50.4)	35 (13.2)		148 (56.1)	116 (43.9)	
Muscularis invasion									
Involved	53 (11.2)	0 (0.0)	3 (5.7)	21 (39.6)	29 (54.7)	< **0.001**	26 (49.1)	27 (50.9)	0.833
None	419 (88.8)	0 (0.0)	116 (27.7)	209 (49.9)	94 (22.4)		212 (50.6)	207 (49.4)	
lamina propria/muscularis involvement (L/M)									
L−/M-	262 (55.5)	0 (0.0)	96 (36.6)	132 (50.4)	34 (13.0)	< **0.001**	147 (56.1)	115 (43.9)	**0.014**
L+/M-	157 (33.3)	0 (0.0)	20 (12.7)	77 (49.0)	60 (38.2)		65 (41.4)	92 (58.6)	
L+/M+	53 (11.2)	0 (0.0)	3 (5.7)	21 (39.6)	29 (54.7)		26 (49.1)	27 (50.9)	
Recurrence									
Present	112 (23.7)	0 (0.0)	23 (20.5)	55 (49.1)	34 (30.4)	0.314	53 (47.3)	59 (52.7)	0.452
Absent	360 (76.3)	0 (0.0)	96 (26.7)	175 (48.6)	89 (24.7)		185 (51.4)	175 (48.6)	
Distant metastasis									
Present	62 (13.1)	0 (0.0)	11 (17.7)	27 (43.5)	24 (38.8)	**0.042**	27 (43.5)	35 (56.5)	0.245
Absent	410 (86.9)	0 (0.0)	108 (26.3)	203 (49.5)	99 (24.1)		211 (51.5)	199 (48.5)	

Values in bold are statistically significant.
H-score indicates histological score.
N (%) shows number of cases and their percentage in brackets.

durations were 82 (SD = 27.8) and 85 (78, 97) months, respectively; with a range of 1–125 months.

3.5. Survival outcomes based on expression of DCLK1

Kaplan–Meier survival was used to analysis and compare DSS and PFS based on the DCLK1 expression (*H*-score) within the respective bladder samples. The mean DSS time for patients with high and low expression of DCLK1 was 104 (SD = 2.4) and 113 (SD = 1.9) months, respectively. The 5-year DSS for patients with samples that had high and low DCLK1 expression was 79 and 89%, respectively ($P = .015$). Kaplan-Meier analysis showed there was a significant difference between DSS and the DCLK1 expression between two groups (Log Rank test; $P = .024$) (Fig. 3). The mean PFS time for patients with high and low expression of DCLK1 was 89 (SD = 1.6) and 97 (SD = 6.1) months, respectively. The results showed there was no significant differences between PFS and the patients with high and low expression of DCLK1 (Log-rank test: $P = .186$). To investigate whether DCLK1 expression was an independent prognostic factor for DSS, and to assess the clinical significance of various parameters that might influence survival outcomes in patients with bladder carcinoma, univariate and multivariate analyses were performed. As summarized in Table 4, DCLK1 expression ($P = .025$), pT stage ($P < .001$), histological grade ($P < .001$), age ($P = .006$), lamina propria involvement ($P = .002$), muscularis invasion ($P < .001$) were significant risk factors affecting the DSS of patients with bladder cancer in univariate analysis. pT stage ($P < .001$), histological grade ($P = .021$), and lamina propria involvement ($P = .001$) were independent prognostic factors in DCLK1 expression, in multivariate analysis. In addition, DCLK1 expression added prognostic value of pT2 stage versus pTa stage ($P = .03$) and high grade versus low grade (P = .02) in patients with bladder cancer. Moreover, we found that DCLK1 protein expression was an independent factor for poor prognosis with respect to DSS in bladder cancer patients ($P = .048$, details in Table 4).

4. Discussion

DCLK1 is a microtubule-associated serine/threonine protein kinase with potential oncogenic roles in the growth, invasion, migration, apoptosis, angiogenesis, and metastasis of tumors (Bailey et al., 2014; Li and Bellows, 2013; May et al., 2010; Mohammadi et al., 2018; Sureban et al., 2015; Wang et al., 2018). It has been recently described as the most specific gastrointestinal CSC markers, and highlighted as a unique colorectal CSC marker that distinguishes CSCs from normal stem cells (NSCs) (Nakanishi et al., 2013). Mohammadi et al. noted the key role played by DCLK1 in invasion, migration, sphere-forming, and apoptosis in colorectal cancer cell lines, is through miR-200c regulation (Mohammadi et al., 2018). Another study reported a direct significant correlation in colorectal cancer between DCLK1 expression with tumor cell progression and metastasis, through the up-regulation of EMT factors, including zinc finger *E*-box-binding homeobox 1 (ZEB1) (Gao et al., 2016). Remarkably, within colon and pancreatic cancers, studies have shown that siRNA-mediated inhibition/knock-down of DCLK1 results in the inhibition of EMT and the suppression of pluripotency,

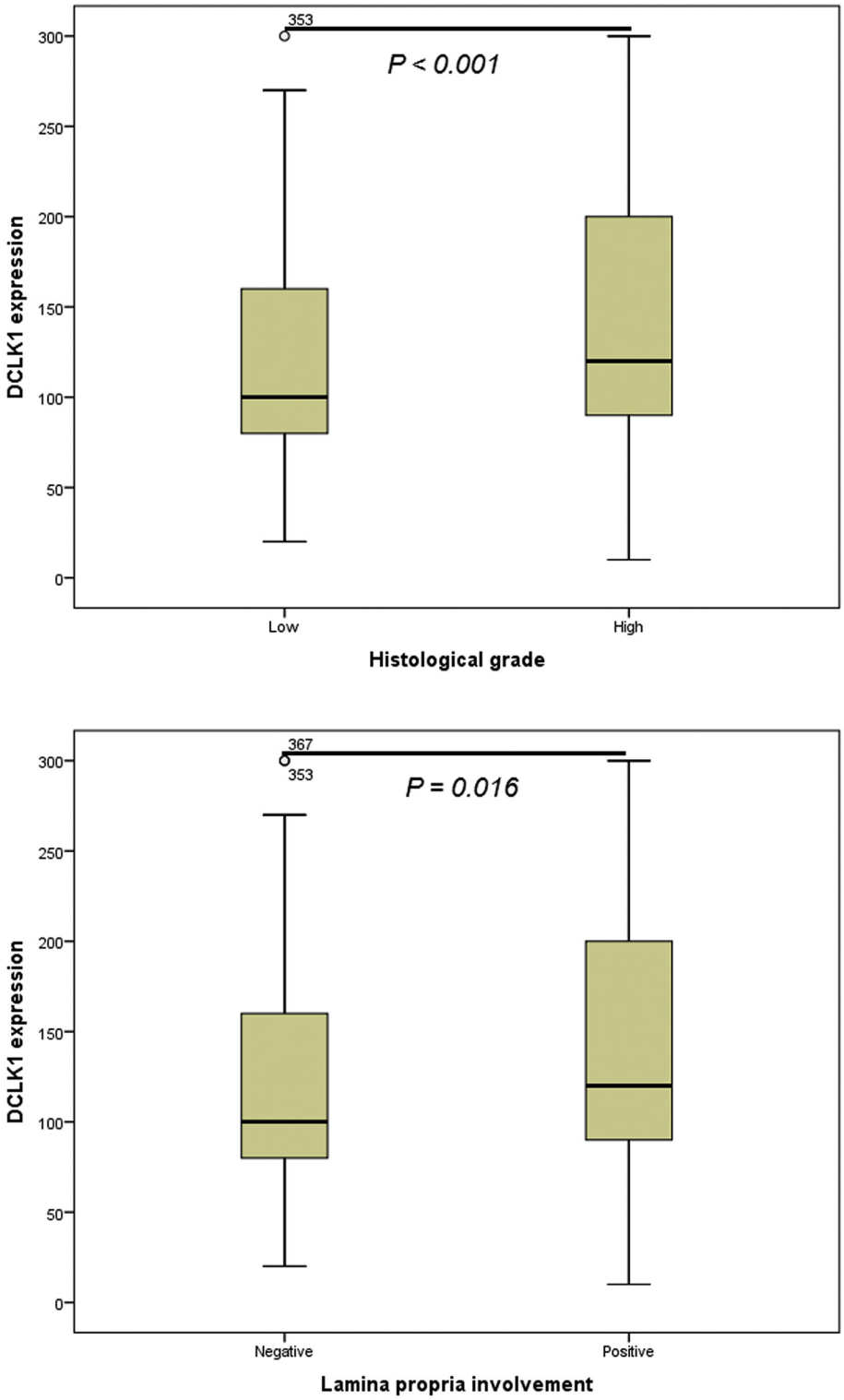

Fig. 2. Box plot analysis of DCLK1 expression levels in histological grade (A) and lamina properia involvement (B) using Mann-Whitney *U* test. On the basis of the standard definitions, each box-plot shows the median (bold line), interquartile lines (box), and outlier observation (circle). The result of the Mann-Whitney *U* test showed that there was a statistically significant association between expression DCLK1 and low and high grades ($P < .001$) and lamina properia involvement or not involved ($P = .016$). The bold line is precisely meant to represent the median DCLK1 expression levels.

angiogenic factors and tumor growth (Sureban et al., 2011a; Sureban et al., 2011b; Sureban et al., 2013; Weygant et al., 2015). These pieces of evidence support the role of DCLK1 as a marker of stemness, also a potential therapeutic marker in gastrointestinal tract malignancies (Sureban et al., 2011b; Sureban et al., 2013; Weygant et al., 2015). Recent evidence suggests an isoform-specific function of DCLK1 in tumor initiation and aggressiveness. Vedeld et al. (2014) reported the silencing of DCLK1 promoter through hypermethylation in 82% of human colorectal cancers, versus no methylation in normal mucosa samples. This likely supports the lower expression of the DCLK1-L isoform, originating from 5′ (α)-promoter, in human colorectal cancer (Marie Vedeld et al., 2015; Vedeld et al., 2014). O'Connell et al. (2015)

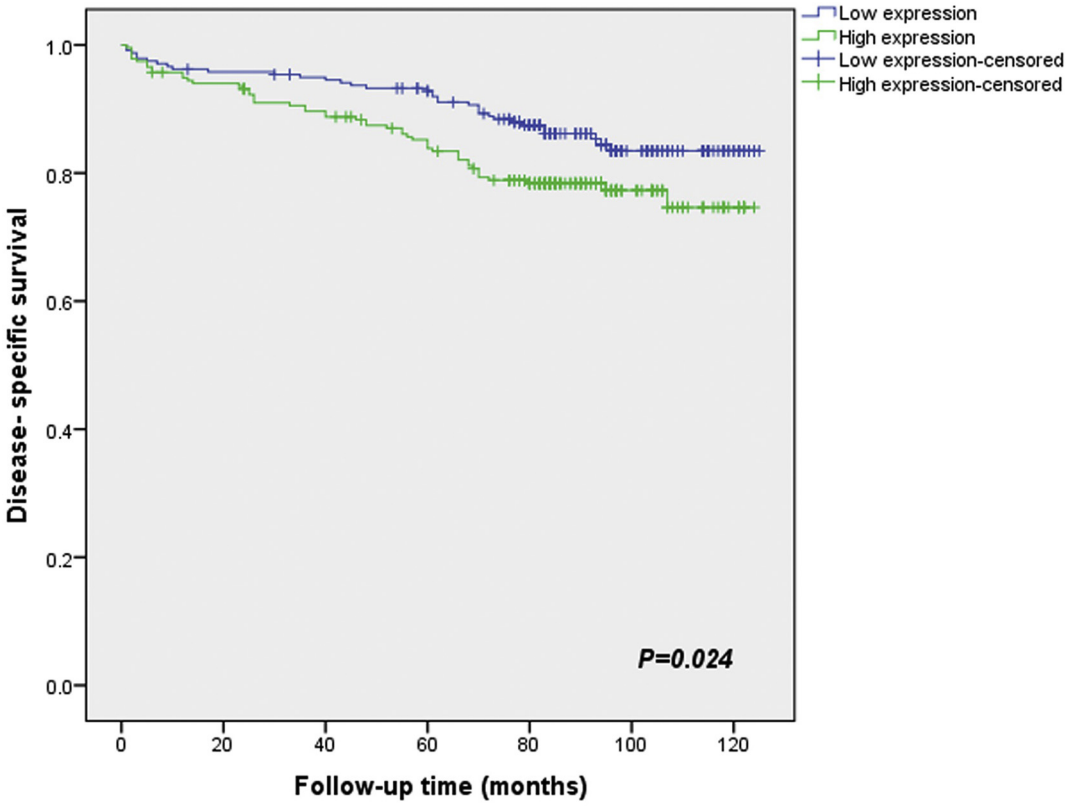

Fig. 3. Kaplan-Meier curve for disease-specific survival (DSS) according to the expression levels of DCLK1 in bladder carcinoma. The results showed that patients with high DCLK1 expression had shorter DSS compared to patients with low DCLK1 expression (P = .024). (expression of DCLK1was grouped into low versus high expression levels).

suggested the alternative transcriptional β-promoter for DCLK1, and its biological function in the higher expression of its corresponding DCLK1-S isoform, in human colorectal cancer. It was also suggested that the hypermethylation of the 5′ (α)-promoter of human DCLK1 gene result in the silencing and loss of the DCLK1-L expression in human colorectal cancer (O'Connell et al., 2015). The antibody used for the purpose of this study detected both DCLK1 L/S isoforms, hence, our findings are likely in line with mentioned studies. This suggests that the higher expression of DCLK1 is likely due to increased DCLK1-S, more so than the DCLK-L isoform.

In our review of the literature, the biological function of DCLK1, as well as clinical utility in urinary tract, including bladder tumors, has received much less attention in comparison to it's role in the GI tract (Zhang et al., 2017). Therefore, the current study was performed to

Table 4
Univariate and multivariate Cox regression analyses of potential prognostic factors for disease-specific survival (DSS) in patients with bladder carcinoma.

Covariate	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
DCLK1 expression				
High versus low	1.642 (1.063–2.534)	**0.025**	1.564 (1.004–2.434)	**0.048**
Age (years)	1.848 (1.197–2.853)	**0.006**	1.419 (0.902–2.231)	0.130
Gender	0.676 (0.359–1.273)	0.225	–	–
Tumor size (cm)	1.397 (0.912–2.141)	0.124	–	–
Histological grade				
High grade versus low grade	2.824 (1.803–4.424)	< **0.001**	2.203 (1.127–4.309)	**0.021**
pT stage		< **0.001**		< **0.001**
pT1 versus pTa	1.600 (0.976–2.624)	0.062	9.469 (1.800–49.817)	**0.008**
pT2 versus pTa	4.441 (2.587–7.626)	< **0.001**	22.711 (4.636–11.25)	< **0.001**
Lamina propria involvement				
Involved versus none	1.993 (1.294–3.071)	**0.002**	0.085 (0.019–0. 386)	**0.001**
Muscularis invasion				
Involved versus none	3.647 (2.242–5.932)	< **0.001**	1.318 (1.102–2.211)	0.150
Recurrence				
Present versus absent	0.034 (0.018–0.063)	< 0.001	-	-
Distant metastasis				
Present versus absent	0.037 (0.023–0.059)	< 0.001	-	-

The variables with *P value* < .05 in univariate analysis were included in multivariable analyses.
HR hazard ratio, *CI* confidence interval.
Values in bold are statistically significant.

evaluate the expression pattern and clinical significance of DCLK1 in the largest series of TCC bladder carcinoma samples ever performed. Our study found that the DCLK1 is overexpressed in bladder tumor cells, compared to normal adjacent tissues. We also found a higher level of DCLK1 expression in higher pT stage, histological grade, and in more invasive tumors. Importantly, DCLK1 expression added prognostic value in patients with bladder cancer who had pT2 stage versus pTa stage as well as high grade versus low grade.

In conjunction with our findings, there are several studies which have pointed the strong clinical value of DCLK1 expression with tumor aggressiveness in colorectal, gastric, and renal cancers (Meng et al., 2013; Mirzaei et al., 2015; Mohammadi et al., 2018; Weygant et al., 2015). Our previous study conducted in a series of 58 fresh and paraffin-embedded colorectal tissues showed a higher expression of DCLK1, both at the protein and mRNA level, in comparison to the normal tissues that were adjacent to the tumor margin (Mirzaei et al., 2015). Moreover, DCLK1 expression had direct association with pT stage and grade in the colorectal samples (Mirzaei et al., 2015). In a series of 172 clear cell renal carcinoma samples, in comparison to adjacent normal samples, mRNA and protein DCLK1 expression was found to have a positive significant correlation with TNM stage (Weygant et al., 2015). Within gastric cancer, a study on 112 samples showed that there was increased DCLK1 expression in tumors with higher pN stage and positive lymphovascular invasion (Meng et al., 2013).

Similar to our findings, a recent study examined the overexpression of DCLK1 in 118 bladder cancer tissues in comparison to 40 normal adjacent tissues. They also found the higher expression of DCLK1 in tumors with higher clinical TNM stage, positive muscularis invasion, positive lymph node metastasis, and distant metastasis (Zhang et al., 2017). Moreover, the increased expression of DCLK1 was found in patients which had lower OS (Zhang et al., 2017). In this study, we also observed that tumors with higher DCLK1expression tend to have a poor prognosis compared to those with low DCLK1expression in DSS. In addition, patients who expressed higher level of DCLK1 had shorter 5-year DSS compared to those with low expression. Moreover, univariate and multivariate analyses showed that the expression of DCKL1 was an independent poor prognostic factor for DSS in bladder cancer patients. Our finding is the first report showing that increased expression of DCLK1 is associated with poor DSS in bladder cancer.

In conclusion, overexpression of DCLK1 was seen in TCCs, in comparison to marginal normal cells. The expression of DCLK1 as a putative CSC marker in a large collection of TCC patients demonstrated that higher DCLK1 expression is associated with increased pT stage and higher histological grade. In addition, increased expression of DCLK1 in tumors with positive lamina propria and lamina propria/muscularis (L/M) involvement indicated that the higher expression of DCLK1 is correlated with more aggressive behavior in TCC tumors. Moreover, in multivariate analysis, pT stage, histological grade, and lamina propria involvement were independent prognostic factors in DCLK1 expression. Additionally, DCLK1 expression was found to be an independent prognostic molecular marker for DSS. As a result, further studies are warranted to define the role of DCLK1 in TCC tumorigenesis, aggressiveness, metastasis, and potential as a targetable agent.

Informed consent

Informed consent was obtained from all individual participants included in the study at the time of sample collection in routine consent forms.

Ethical approval

The study was approved by the Iran University of Medical Sciences Human Research Ethics Committee in Iran (Ref No: IR.IUMS. FMD.REC 1396.9311100003). All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of interest

The authors declare that they have no conflict of interest.

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