Cytoplasmic Expression of DCLK1-S, a Novel DCLK1 Isoform, Is Associated with Tumor Aggressiveness and Worse Disease-Specific Survival in Colorectal Cancer

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Primary research

Keywords: Colorectal cancer, DCLK1-S, Polyclonal antibody, Immunohistochemistry, Tissue microarray

Posted Date: March 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-148922/v2>

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Version of Record: A version of this preprint was published at Cancer Biomarkers on December 23rd, 2021. See the published version at <https://doi.org/10.3233/CBM-210330>.

Abstract

Background: Oncogenic role of doublecortin-like kinase 1 (DCLK1) as a putative cancer stem cell (CSC) marker has been clarified in colorectal cancer (CRC). Isoform-specific function of DCLK1 has highlighted key role of the DCLK1-S (short isoform) in tumor maintenance, progression, and invasion. Considering the lack of commercial anti-DCLK1-S antibody suitable for immunohistochemical (IHC) application, this study was conducted to produce and validate an anti-DCLK1-S polyclonal antibody in order to specifically evaluate expression pattern and clinical significance of short isoform of DCLK1 in colorectal cancer tissues.

Methods: Rabbit immunization was performed against a synthetic peptide corresponding to the published six specific amino acid sequences of DCLK1-S, and production of antibody was evaluated by enzyme-linked immunosorbent assay (ELISA). After IHC assessment of the purified anti-DCLK1-S polyclonal antibody, it was used to undertake a definitive study for determining prognostic significance of DCLK1-S expression in a well-defined tissue microarray (TMA) series including 348 CRC and 51 adjacent normal tissues with a follow-up period of 108 months.

Results: Positive immunoreactivity of DCLK1-S was found in 84.2% of CRC samples. Cytoplasmic expression was the main localization of DCLK1-S compared to nuclear and membranous area of tumor cells. Expression of DCLK1-S in CRC samples was significantly higher compared to adjacent normal samples ($P < 0.001$). A positive significant association was found between high cytoplasmic expression of DCLK1-S and advanced tumor, nodes, and metastases (TNM) stage ($P < 0.001$) as well as the increased tumor differentiation ($P = 0.02$). Moreover, the patients with CRC whose tumors showed higher cytoplasmic expression of DCLK1-S had worse disease-specific survival (DSS) (log-rank test, $P = 0.03$) and 5-year DSS rate ($P = 0.01$). Additionally, the improved prognostic value was seen in the patients with CRC with high DCLK1-S expression versus moderate expression (HR: 2.70, 95% CI: 0.98-7.38; $p = 0.04$) by multivariate analysis.

Conclusions: Our findings strongly supported that DCLK1-S isoform may play a crucial role in invasion, tumor aggressive behavior, and worsened DSS of the patients with CRC. Importantly, high cytoplasmic expression of DCLK1-S compared to moderate expression could be considered as an independent prognostic factor influencing DSS. Taken together, DCLK1-S can be a candidate as a promising prognostic and targeted-therapeutic indicator for effective treatment of CRC.

Background

Colorectal cancer (CRC) is the fourth most frequently diagnosed cancer and leading cause of global death [1]. Despite developments in anti-cancer treatments, most CRC cases are resistant to conventional cancer therapies. Surgery, radiotherapy, chemotherapy, and targeted therapy are used as the current treatment for the patients with CRC. Combining these traditional methods with new targeted-therapy strategies could increase survival rate of the patients with CRC [2]. Therefore, identifying biomarkers can be considered an essential step for improving targeted molecular therapies (immunotherapy) and increasing survival of the patients with cancer. Considering crucial role of cancer stem cells (CSCs) in tumorigenesis, tumor invasion, and drug-resistance, many efforts have been made to discover specific markers in order to target CSCs in various cancers including CRC [3-6]. Among various markers used for identification of CRC-CSCs, doublecortin-like kinase 1 (DCLK1), as a putative CSC marker has been shown to have a crucial oncogenic role in various *in-vivo* and *in-vitro* experiments, particularly in pancreatic and colon cancers [5, 7-10] so that, DCLK1 is called as a well-known CRC-CSC marker distinguished cancer cells from normal cells in Apc Min/+ mice [7].

In the recent decades, many experiments have focused on biological function of two different transcripts of DCLK1; long isoform (DCLK1-L, NM_004734.4) and short isoform (DCLK1-S, NM_001195415.1) with molecular weight of ~80–82 and ~45–50 kDa, respectively [9, 11, 12]. Evidence has suggested more than 98% of similarity between DCLK1-L and DCLK1-S isoforms at C-terminal end of both proteins, while the only difference in the sequence of six amino acids was found at N-terminal end of the DCLK1-S protein [9, 10]. Further molecular evidence regarding isoform-specific function of DCLK1 in tumor initiation, proliferation, and aggressiveness has shown oncogenic role of DCLK1-L at the early stages and DCLK1-S in progression and invasion of CRC and pancreatic cancer [8, 13, 14]. Epigenetically, due to silencing of 5'(α)-promoter of DCLK1 gene as an early event during CRC carcinogenesis, overexpression of DCLK1 in CRC experiments was a vague issue [9]. This controversy was addressed when oncogenic biological role of an alternative transcriptional β -promoter of DCLK1 was clarified [9]. O'Connell et al., suggested that although DCLK1-S isoform originated from β -promoter may have the primary CSC characteristics of DCLK1 protein, DCLK1-L could play a leading role in identification of normal or low-stage vs. high-stage CRC cells [9, 15]. They showed higher expression of DCLK1-S in the patients with CRC with shorter overall survival by quantitative real-time polymerase chain reaction (qRT-PCR) technique and ultimately, they suggested DCLK1-S as a specific CRC biomarker for characterization of high-risk patients after colonoscopy [9, 10]. Other molecular experiments have reported overexpression of DCLK1-S vs. DCLK1-L in tumor cells compared to normal cells influenced by forkhead-box-D3 (FOX D3) gene in regulation of DCLK1-S in colorectal carcinomas [16], although more investigations are required to define specific biological difference of these two isoforms yet.

In our previous research, expression pattern and prognostic significance of DCLK1 have been studied in colorectal, gastric, and bladder carcinomas [17-21] using available commercial anti-DCLK1 antibodies that can detect C-terminal domain of both DCLK1-L/S isoforms or only N-terminal end of DCLK1-L isoform, while role of the main isoform to overexpress DCLK1 has remained unknown. To the best of our knowledge, there is no commercial anti-DCLK1-S antibody, and also no study has been previously performed to investigate prognostic significance of DCLK1-S on tumor tissues, particularly in CRC by immunohistochemistry (IHC). Therefore, for addressing these gaps, the present study was conducted to generate an anti-DCLK1-S-specific polyclonal antibody elicited by six amino acids synthetic peptide that has been reported as the only different form in the sequence homology epitopes of both DCLK1 isoforms (DCLK1-L and DCLK1-S)[10].

In the current study, for the first time, membranous, cytoplasmic, and nuclear localization of DCLK1-S elicited by polyclonal anti-DCLK1-S antibody, generated in our laboratory was evaluated in a large number of formalin-fixed, paraffin-embedded (FFPE) series of CRC samples by tissue microarray (TMA)-based IHC analysis. Prognostic significance of short isoform of DCLK1 (DCLK1-S) was also investigated in this series of the patients with CRC.

Methods

Production of anti-DCLK1-S-specific polyclonal antibody

Polyclonal antibody to DCLK1-S was generated in our laboratory, as described previously [22-24]. A synthetic peptide corresponding to the published amino acid sequence of DCLK1-S isoform (MLELIE) was selected as immunogen [10]. A cysteine residue was added to C-terminal of peptide (NH₂-Met-Leu-Glu-Leu-Ile-Glu-Cys-COOH) for conjugation with Imject Maleimide-activated mckeyhole limpet hemocyanin (KLH) (Thermo Scientific, Rockford, IL, USA) and used for immunization of two female New Zealand White Rabbits purchased from Pasteur Institute of Iran. To assess conjugation efficacy, the peptide was also conjugated with Imject Maleimide-activated bovine serum albumin (BSA) (Thermo Scientific, Rockford, IL, USA). Conjugation efficacy was investigated by analysis of electrophoretic pattern of the BSA-conjugate [22]. Production of DCLK1-S specific antibody was evaluated by enzyme-linked immunosorbent assay (ELISA) [25]. Anti-DCLK1-S specific antibodies were purified by the peptide coupled to CNBr-activated Sepharose-4B. Purity and reactivity of the purified antibodies were assessed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA techniques, respectively. Immunoreactivity pattern of the generated antibody was compared with available commercial anti-DCLK1 antibody; ab31704 (which is commonly used to detect long and short DCLK1 isoforms; Abcam, UK) in CRC tissues by IHC method.

Indirect ELISA

Reactivity of rabbits' sera as well as the purified anti DCLK1-S antibodies was tested by ELISA [22]. Briefly, coating was performed using 10 µg/ml of the peptide diluted in phosphate-buffered saline (PBS, 0.15 M, pH 7.2), which was incubated at 37 °C for 1 h followed by overnight incubation at 4 °C. After several washes, plates were blocked using 2.5% skimmed milk at 37 °C for 1 h and then, serial dilutions of rabbits' sera or the purified antibodies were added. Following serum incubation (37 °C for 1.5 h) and washing, a horseradish peroxidase (HRP) conjugate of sheep anti-rabbit immunoglobulin (Ig) antibody (Avicenna Research Institute, Tehran, Iran) diluted 1:1500 in phosphate buffered saline-Tween 20 (PBS-T) was added to the wells (37 °C, 1.5 h). Following washing, substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (Pishtaz Teb Zaman, Iran) was added and optical density (OD) values were measured at wavelength of 450 nm on a microplate reader (Synergy HTXMulti-Mode Reader, BioTek, USA).

Sample collection and patients characteristics

FFPE series of 385 CRC samples and 70 matched adjacent normal tissues were collected from university hospitals including Hasheminejad, Rasool Akram, and Firoozgar during 2010 - 2019 in Tehran, Iran. Clinicopathological information including age, gender, tumor size, tumor differentiation, vascular and neural invasion, lymph node involvement, tumor, nodes, and metastases (TNM) staging, distant metastasis, and tumor recurrence was collected from the patients' documents. None of the patients with CRC in this study had received neoadjuvant treatment before surgery. Size cutoff and TNM stage of the tumors were determined according to the American joint committee on cancer/ union for international cancer control (AJCC/UICC) and TNM staging system, respectively [26, 27]. The patients' data were kept anonymous in all steps of the study. Interval from the date of surgery to the date of death by CRC was used for disease-specific survival (DSS) analysis. Moreover, progression-free survival (PFS) was defined as the interval between the primary surgery and the last follow-up visit if the patient showed no evidence of disease, tumor recurrence, or distant CRC metastasis. The current study was approved by the Human Research Ethics Committee of the Iran University of Medical Sciences, Tehran, Iran (Ref No: IR.IUMS.REC 1396.32186). All the procedures performed in this study were in accordance with the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all individual participants, parents or legally authorized representatives of participants at the time of sample collection with routine consent forms.

TMA construction

Colorectal tissue arrays were prepared and constructed by a TMA instrument (Minicore; ALPHELYS, Plaisir, France), as described previously [4, 28, 29]. Three representative areas of the tumors were pointed on hematoxylin & eosin (H & E) slides of CRC samples by pathologist. Different regions of each tumor were punched into TMA recipient blocks in three copies. Based on TMA studies, each core would denote to the whole tissue's staining pattern with 90% of accuracy, while using two cores or more could increase this accuracy by 95-99%, respectively [30]. In this study, for overcoming heterogeneity in antigen expression and increasing accuracy and validity of the analysis, three cores were evaluated from each tissue sample. Mean score of the three cores was calculated as the final score for IHC analysis of DCLK1-S.

Immunohistochemistry

Immunostaining of CRC slides (whole tissue and TMA slides) was performed by standard chain polymer-conjugated antibody (EnVision), as described previously [4, 19, 31]. Briefly, following de-waxing at 60°C for 30 min and rehydration steps, heat-activated antigen was retrieved by autoclaving the sections in citrate buffer (10 mM, pH 6) at 95°C for 11 min. Endogenous peroxidase activity and non-specific binding sites were blocked by H₂O₂ (3 %) and 5 % normal sheep serum diluted in protein block (Dako, CA, USA), respectively. The sections were incubated overnight using a polyclonal anti-DCLK1-S antibody generated in our laboratory (0.75 µg/ml) at 4 °C. Then, the sections were treated with the secondary antibody, TMMouse/Rabbit PolyVue HRP/DAB Detection kit (standard EnVision-HRP kit, Bio pharmadx), at room temperature (RT). The 3, 3'-diaminobenzidine (DAB) substrate as a chromogen was used to visualize immune signals. The sections were counterstained with hematoxylin for 5 min, were dehydrated and finally, were mounted. Humans CRC whole tissue was selected as positive control for DCLK1-S. In negative reagent controls, the primary antibody was replaced by non-immune rabbit IgG as isotype control to confirm specific bindings of anti-DCLK1-S antibody. In addition, in all the experiments, specificity of the produced antibodies was evaluated by blocking antibodies with saturating concentration (1:100 molar ratio) of immunizing peptide before incubating the slides with the primary antibody. The primary antibody was also replaced by tris buffer saline (TBS, pH: 7.4) as negative control to confirm the absence of non-specific bindings of the secondary antibody. Normal and cancerous tissues of ovary [32], testis, and skin were also used as negative control tissues based on protein atlas databases for further confirmation of specificity of our polyclonal antibody. Digital images were captured by an H550S microscope (Nikon, Japan) and a Digital sight DS-LS camera (Nikon, Japan).

Evaluation of immunostaining

Scoring step was performed semi-quantitatively in a coded manner, by two pathologists (MA and MR) who were blinded to clinical and pathological parameters (as described previously) [33], and a consensus agreement was achieved. Given an agreement to the final score of DCLK1-S expression, the general distribution of the tumor cells was assessed at 10× magnification. Then, positive cells were evaluated semi-quantitatively at higher magnifications, and final scores were given, respectively. Percentage of positive cells, intensity of the staining, and histochemical score (*H*-score) were three scoring methods by which expression pattern of DCLK1-S was evaluated. Intensity of immunostaining was divided into four groups; 0(negative), 1(weak), 2(moderate), and 3(strong) staining. Percentage of positive cells was valued semi-quantitatively and was scored as 0–100%. The *H*-score was obtained by multiplying intensity (0–3) by percentage scores (0–100%), which led to generation of the final scores of 0-300. In the present study, *H*-score was divided into three groups: 0–100 (low expression), 101–200 (moderate), and 201–300 (high expression) [34].

Statistical analysis

SPSS software version 22 (IBM Corp, USA) was used for statistical analysis. Pearson's χ^2 and Spearman's correlation tests were performed to analyze significance of association and correlation between DCLK1-S protein expression and clinicopathological features. Moreover, Kruskal–Wallis and Mann–Whitney U tests were carry out to compare between the groups. DSS and PFS analysis were used to adopting the Kaplan–Meier method, with along the log-rank test to estimate curves between the groups with 95% confidence interval (CI). To determination the variables were influenced DSS or PFS, Cox (Proportional Hazards) Regression was performed. All the categorical data were described by N (%), a valid percentage, and quantitative data were exhibited as mean, standard deviation (SD), median, and quartile (Q1, Q3). A P-value of <0.05 was considered as statistically significant.

Results

1. Production of Antibody and Characterization

1.1. Production of Anti-DCLK1-S Antibody

Efficacy of conjugation and immunization was evaluated by SDS-PAGE analysis of the BSA-conjugate and ELISA test, respectively. Smear pattern of SDS-PAGE and the absence of free peptide showed effective conjugation of BSA-peptide (supplementary Fig.1 (A)). Immunoreactivity of the antibodies was confirmed by ELISA test. The results showed high immunoreactivity of the antibodies reaching to plateau as low as an antibody concentration of 1.25 ng/ml (supplementary Fig.1 (B)).

1.2. Validation of Anti-DCLK1-S Antibody

Immunoreactivity of anti-DCLK1-S was evaluated by IHC method on CRC whole tissues as positive control samples and humans' normal and cancerous tissues of testis, ovary, and skin as negative control samples. In addition, isotype control and primary antibody saturated with immunizing peptide were used as negative reagent controls in all the experiments. The results clearly showed specific pattern of immunoreactivity in CRC tissues compared to negative control tissues (Fig. 2 and supplementary Fig. 2). The antibody could strongly recognize DCLK1-S in CRC samples, and localization was restricted to cytoplasmic area and partially in cell membrane and nucleus area of CRC samples, (Fig. 1). No staining was observed in humans' normal and cancerous tissues of testis, ovary, and skin, demonstrating specific immunoreactivity of the produced anti-DCLK1-S antibody (supplementary Fig. 2).

The commercial anti-DCLK1 antibody, which can recognize C-terminal end of both long and short isoforms of DCLK1 (anti-DCLK1-L/S antibody; ab31704) was used to compare expression pattern of DCLK1-L/S vs. DCLK1-S. Results showed no difference in pattern of expression using the generated anti-DCLK1-S antibody and the commercial anti-DCLK1-L/S antibody, as shown in supplementary Fig.3. No staining was also observed in negative control sections incubated by pre-adsorbed or substituted by pre-immune-purified rabbit IgG as primary antibody (Fig.2).

2. Immunohistochemical Evaluation of Anti-DCLK1-S Antibody in CRC Tissues

2.1. Study Population

Considering technical problems during TMA construction and IHC staining, among all 385 collected CRC samples and 70 adjacent normal tissues, 348 CRC and 51 adjacent normal tissue samples remained for statistics analysis of DCLK1-S expression. Patients had a mean age of 60 ± 14.4 years old, and males accounting for 51.8% of our study population. Mean tumor size was equal to 5 ± 2.2 cm (ranged from 2 to 14 cm), of which 236 (67.6%) were less than 5 cm. Regarding available tumor differentiation data of 346 patients; 121(35%) patients had well, 192(55.5%) patients had moderate, and 33(9.5%) patients had poor tumor differentiation. Regarding available data of TNM stage for 296 samples, 52 (17.6%) cases had stage I, 133 (44.9%) cases had stage II, 100 (33.8%) cases had stage III, and 11 (3.7%) cases had stage IV. Vascular, neural, and lymph node involvement was found in 59 (17.2 %), 67 (19.5%), and 118 (34.3 %) of cases, respectively. Table 1 summarizes all the clinicopathological characteristics of the study population.

2.2. Expression of DCLK1-S in the Patients with CRC and Adjacent Normal Samples

Results of IHC analysis showed that majority of CRC samples (293/348, 84.2%) had a positive DCLK1-S expression. All the positive samples represented cytoplasmic localization of DCLK1-S in tumor cells' area (293/293, 100%), which 9.5% (28/293) of them showed both cytoplasmic and nuclear expressions and 4.7% (14/293) of them showed both cytoplasmic and membranous expression of DCLK1-S (Fig. 1). In terms of intensity, among 348 CRC samples, 36 (10.3%) of them had strong, 77 (22.2%) of them had moderate, 180 (51.7%) of them had weak, and 55 (15.8%) of them had negative staining (Fig. 2). *H*-score was classified as low ($100 \leq$), moderate ($100-200$), and high ($201 \geq$) histochemical reactivity. Among 348 cases, 255 (73.3%) of them showed low, 83 (23.8%) of them showed moderate, and 10 (2.9%) of them had high immunoreactivity of DCLK1-S. There was a statistically significant difference in expression of DCLK1-S (in terms of intensity of staining, percentage of positive cells, and *H*-score; $P < 0.001$) between CRC and adjacent normal samples. The highest expression of DCLK1-S was found only in CRC samples compared to adjacent normal tissue samples (Fig. 3). In terms of intensity, none of the adjacent normal tissues demonstrated strong reactivity of DCLK1-S, but moderate, weak, and negative intensity was found in 3 (5.9%), 20 (39.2%), and 28 (54.9%) of tissues. Moreover, in terms of *H*-score, 48/51 (94.1%) of samples displayed low scoring pattern, only 3/51 (5.9 %) of cases showed a moderate score of DCLK1-S expression, and no high scoring pattern was detected in adjacent normal tissues (Table 2).

2.3. Cytoplasmic Expression of DCLK1-S Was Associated with Tumor Aggressiveness in CRC Tissues

The results of Pearson's χ^2 test revealed a statistically significant association between overexpression of DCLK1-S and advanced TNM stage ($P < 0.001$) as well as the increased tumor differentiation ($P = 0.03$). Furthermore, the results of Spearman's correlation test exhibited a direct significant correlation between cytoplasmic DCLK1-S expression and TNM stage ($P < 0.001$) and also tumor differentiation ($P = 0.02$). Moreover, the results of the Kruskal–Wallis test indicated a statistically significant difference between median cytoplasmic expression level

of DCLK1-S and tumor differentiation ($P=0.04$). Mann–Whitney U test also showed a statistically significant difference in median expression level of cytoplasmic DCLK1-S between poor and moderate tumor differentiation ($P=0.04$). Results of statistical analysis did not show any significant association between DCLK1-S expression and other clinicopathological characteristics including age, gender, tumor size, vascular and neural involvement, distant metastasis, and tumor recurrence. All findings are available in Table 1.

2.4. Cytoplasmic Expression of DCLK1-S Was a Poor Prognostic Factor of Disease-Specific Survival (DSS) in CRC Tissues

Among 348 CRC samples included in this study, 235 (67.5%) samples had a history of tumor recurrence, distant metastasis, or cancer-related death events. Distant metastasis and tumor recurrence occurred in 67 (28.5%) and 72 (30.6%) of patients, respectively, while in 80 (34%) of patients, metastasis and recurrence was not observed. During the follow-up period, cancer-related death and the other causes of death were documented in 67 (90.5%) and 7 patients (9.5%), respectively. Mean and median follow-up times were equal to 43.5 (SD = 29.7) and 38 (21, 38) months, with a range of 1–108 months.

The results of Kaplan–Meier survival analysis represented that the CRC patients with high cytoplasmic expression of DCLK1-S had shorter DSS compared to those with moderate and low cytoplasmic DCLK1-S expression (log-rank test, $P = 0.03$) (Fig.4(a)). Mean $\pm$ SD of DSS time was equal to 48 ± 14 , 69 ± 6 , and 81 ± 3.5 , for the patients with high, moderate, and low cytoplasmic DCLK1-S expression, respectively. There was no significant difference between PFS and the patients with high, moderate, and low cytoplasmic expression of DCLK1-S (log-rank test, $P = 0.17$) (Fig.4 (b)). Mean $\pm$ SD of PFS time was equal to 48 ± 14 , 67.7 ± 6 , and 74 ± 3.6 , for the patients with high, moderate, and low cytoplasmic DCLK1-S expression, respectively.

Moreover, 5-year DSS was obtained as 50, 62, and 74% , for the patients with high, moderate, and low level of cytoplasmic expression of DCLK1-S , respectively (mean $\pm$ SD of DSS time was equal to 48 ± 14 , 69 ± 6 , and 81 ± 3.5 , for the patients with high, moderate, and low cytoplasmic expression of DCLK1-S , respectively $P= 0.01$). As demonstrated in Table 3, the results of univariate analysis showed that cytoplasmic expression of DCLK1-S ($P=0.04$), age ($P= 0.04$), tumor differentiation ($P = 0.01$), TNM stage ($P= 0.02$), vascular invasion ($P= 0.02$), neural invasion ($P= 0.03$), and lymph node involvement ($P=0.001$) were significant risk factors influencing DSS as confirmed by univariate Cox regression analysis. Moreover, prognostic value was increased in high cytoplasmic expression of DCLK1-S vs. moderate expression of DCLK1-S (HR: 2.70, 95% CI: 0.98-7.38; $p =0.04$) in the patients with CRC in multivariate analysis. Therefore, high cytoplasmic expression of DCLK1-S compared to moderate expression could be considered as an independent prognostic factor of DSS in multivariate analysis.

Discussion

DCLK1, a putative CSC marker is a member of microtubule-associated proteins whose biological function has been identified in different human cancers, particularly in colorectal and pancreatic carcinomas [8, 10, 35]. The previous studies have also reported that suppressing DCLK1 had a significant direct correlation with tumor invasiveness and drug resistance in renal clear cell cancer and pancreatic cancer [36, 37].). In CRC, upregulation of DCLK1 is significantly associated with tumor progression, aggressiveness, metastasis, and poor prognosis [38]. Therefore, oncogenic role of DCLK1 in a wide range of biological studies has suggested prominence of the DCLK1 marker as a promising molecular target in treatment of CRC. From this point, finding distinguished CSC markers which have not been identified on normal stem cells play a fundamental role in targeted-therapy strategies and tumor eradication. Nakanishi et al., revealed CSC characteristics of DCLK1 in intestinal tumor cells but not in normal stem cells *in-vivo* [7]. Vreugdenhil et al., cloned different transcripts of DCLK1 and discovered two distinguishing alternative splicing variants of DCLK1 transcripts; DCLK-short and DCLK-long [39]. The previous studies have also highlighted isoform-specific function and localization profile of these transcripts in Wistar rats' brains [12]. Interestingly, epigenetic differences have discovered that DCLK1-S isoform (~47 KDa), originated from β -promoter plays a significant role during colon carcinogenesis by the response to oncogenic pathways, while DCLK1-L isoform (~82 KDa) could be detected in the early stage of CRC and normal colonic mucosal samples vs. high-stage tumors [9, 15]. These investigations have strongly supported that DCLK1-S as an isoform of DCLK1 is identified as a requisite factor in tumor invasion, proliferation, and metastasis potential of CRC cells [15].

Although, many preliminary studies have confirmed clinical value of DCLK1 expression in tumor progression and aggressiveness, the available commercial anti-DCLK1 antibodies widely used in the previous studies could detect sequence homology epitopes of both isoforms, DCLK1-L and DCLK1-S, or only DCLK1-L isoform; hence, specific implication of DCLK1-S has still remained largely unknown.

In our previous works, oncopathological value of DCLK1 expression was evaluated in various cancers including colorectal, bladder, and gastric carcinomas; results of which were in line with other studies [17, 19, 36, 40-43]. In a comprehensive study on 472 bladder cancer cases, an increase was found in expression of DCLK1 (DCLK1-L/S) that associated with more aggressive tumors, advanced stages, and poorer DSS in the patients with bladder cancer [19]. Additionally, results of our previous study on local and circulating DCLK1 (DCLK1-L) in

58 fresh CRC tissues and their correspondent normal margins demonstrated significantly higher expression of DCLK1 in the patients with CRC having advanced-stage and higher tumor grade in both protein and mRNA expression levels [41]. We have recently reported highly significant expression of DCLK1 (DCLK1-L/S) in CRC cases compared to adenomatous and non-adenomatous colonic polyps, as well as a positive significant correlation between DCLK1 expression and tumor size, tumor differentiation, and lymph node involvement [20]. Other clinical investigations have also represented that higher expression of DCLK1 (DCLK1-L/S) had a significant positive correlation with tumor invasion, metastasis, and shorter survival in the patients with renal clear cell carcinoma and bladder cancer [36, 43]. However, it is important to state that the commercial anti-DCLK1 antibodies used in these studies can only recognize C-terminal end of DCLK1-L/S protein, or only N-terminal end of DCLK1-L isoform; thus, further studies are required to investigate biological role of DCLK1-S in tumor progression and invasiveness.

Since, the absence of the commercial anti-DCLK1-S antibody was the primary obstacle for assessing this molecule at the protein level, in the present study; a polyclonal specific antibody against DCLK1-S was successfully developed in our laboratory. This will provide further opportunities to investigators to obtain more accurate and valuable findings related to DCLK1-S localization and its clinical significance as the unique DCLK1 isoform responsible for invasiveness and aggressive behavior in various tumors by IHC method.

Our findings demonstrated that majority of CRC samples with positive expression of DCLK1-S had pronounced cytoplasmic localization, besides partially nuclear and very low membranous expression patterns of S-isoform. In parallel, the results of immune electron microscopy (IEM) have confirmed the presence of S-isoform in cytosol, mitochondrial, and cell membrane fractions of HCT-116 and COLO205-S-GFP cell lines, unlike L-isoform that was present mainly in cell membrane fractions of HEK293 cells only expressing L-isoform [10]. Other clinical studies used anti-DCLK1-L/S or anti DCLK1-L antibodies have also reported mainly cytoplasmic and membranous localization of DCLK1 in gastric, colorectal, and bladder carcinoma tissues [18, 19, 33, 43], meanwhile in the current study, nucleus localization of DCLK1-S was shown in tumor cells' area.

Besides, O'Connell et al., suggested that DCLK1-S was the main DCLK1 isoform expressing in humans' CRC cell lines vs. DCLK1-L markedly expressing in normal colorectal cell lines thus, DCLK1-S can be used as a promising prognostic and targeted-therapeutic marker in treatment of CRC [9]. Other studies have demonstrated higher expression of DCLK1-S in polyps from high-risk vs. low-risk patients and also downregulation of DCLK1-L/FOXD3 in polyps from high-risk compared to low-risk patients, indicating the potential suppressor function of FOXD3 in regulation of DCLK1-S [16]. The existence of FOXD3 in normal or low-risk polyps inhibited expression of DCLK1-S, while methylation of FOXD3 in cancerous cells can cause overexpression of DCLK1-S in high-risk cases.

Our comparative immunostaining findings showed similar pattern of immunoreactivity by anti-DCLK1-S antibody and anti DCLK1-L/S antibody, which both represented strong staining of DCLK1 in CRC tissues. Similar staining pattern in both antibodies may be due to the fact that DCLK1-S is predominant isoform in CRC tissues. Although, further studies are required to show different expression of these two isoforms.

The current study provided novel and interesting insights into expression pattern and prognostic significance of DCLK1-S in a well-defined series of CRC tissues. Results of IHC analysis revealed a significant upregulation of cytoplasmic DCLK1-S expression in CRC samples compared to adjacent normal tissues. Furthermore, a significant association was found between high expression of DCLK1-S and advanced TNM stage, the increased tumor differentiation, and poor DSS in these cases. Consistent with our study, there is only one study pointed a significant clinical value of DCLK1-S expression associated with tumor aggressiveness at mRNA level in the patients with CRC [9]. Additionally, our results showed that 5-year DSS was equal to 50, 62, and 74%, for the patients with high, moderate, and low cytoplasmic expression of DCLK1-S, respectively.

Conclusions

Taken together, for better understanding on specific role of the main DCLK1 isoform in aggressiveness of CRC tumors, herein, a specific polyclonal anti-DCLK1-S antibody was produced identified only by six particular amino acids related to short isoform and its specificity was validated by IHC method in CRC samples. Our findings strongly supported critical role of DCLK1-S as the DCLK1 isoform, which led to tumor aggressiveness and worsened DSS in CRC samples. Importantly, high cytoplasmic expression of DCLK1-S protein compared to moderate expression was considered as an independent poor prognostic factor of DSS in these series of CRC cases. Hence, additional investigations and larger trials on the potential therapeutic role of DCLK1-S as a novel CRC agent can shed new lights on treatment of the patients with CRC.

Abbreviations

CRC: Colorectal cancer; CSC: Cancer stem cell; DCLK1: doublecortin-like kinase 1; DCLK1-L: DCLK1 long isoform; DCLK1-S: DCLK1 short isoform; FOXD3: Forkhead-Box-D3; FFPE: formalin-fixed, paraffin-embedded; IHC: Immunohistochemistry; TMA: tissue microarray; KLH: Imject Maleimide-activated mckeyhole limpet hemocyanin; BSA: Imject Maleimide-activated bovine serum albumin; ELISA: enzyme-linked immunosorbent assay; SDS-PAGE: sodium dodecyl-sulfate polyacrylamide gel electrophoresis; HRP: horseradish peroxidase; Ig: immunoglobulin; PBS-T: phosphate buffered saline-Tween20; TMB: 3,3',5,5'-tetramethylbenzidine; TNM: tumor, nodes, and metastases staging; H & E; hematoxylin & eosin; H-score: histochemical score; IEM: immune electron microscopy; OD: optical density; HRs: Hazard ratios; CIs: Confidence intervals; PFS: progression-free survival; DSS: Disease-specific survival.

Declarations

Ethics approval and consent to participate

The current study was approved by the Human Research Ethics Committee of the Iran University of Medical Sciences, Tehran, Iran (Ref No: IR.IUMS.REC 1396.32186). All the procedures performed in this study were in accordance with the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all individual participants, parents or legally authorized representatives of participants at the time of sample collection with routine consent forms.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare no competing interests.

Funding

This study was supported by an Educated Scholar Grant from the National Institute for Medical Research Development (NIMAD) (Grant #982625). The current study was part of PhD thesis with Grant Number: 96-03-126-32186, which carried out at Oncopathology research center in Iran University of Medical Sciences.

Authors' contributions

Z.M. and R.Gh. designed and supervised the work; E.K. wrote the manuscript, did literature search, data acquisition and statistical analysis, constructed TMA blocks and performed all experiments. L.S. analyzed interpreted the data, helped to prepare the Tables as well as contributed to edit of the manuscript. M.R. scored TMAs slides after immunohistochemically staining and helped to prepare the Fig. 2. L.E. helped to prepare the blood sampling of the rabbits in animal experiment section. M.R. helped to literature search and prepare the Fig.1. M.A. contributed in manuscript edit, and data analysis. All authors read and approved the final manuscript.

Acknowledgment

We are grateful of our colleague at Iran University of Medical Sciences who provided insight and expertise that greatly assisted the research. Apart from, it must be declared that the authors received no specific funding for this work.

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Tables

Table 1. The association between cytoplasmic DCLK1S expression and clinicopathological parameters of colorectal cancer (CRC) samples (Intensity of staining and H-score)										
Patients and tumor characteristics	Total samples	Intensity of staining N (%)				<i>P-value</i>	H-score N (%)			<i>P-value</i>
	N (%)	Negative (0)	Weak (1+)	Moderate (2+)	Strong (3+)		Low (0-100)	Moderate (100-200)	High (200-300)	
Colorectal cancer										
Mean age, years (Range)	176(52.9)	27(15.3)	85(48.3)	46(26.2)	18(10.2)		120(68.2)	50(28.4)	6(3.4)	
≤Mean age	157(47.1)	28(17.8)	85(54.1)	26(16.6)	18(11.5)	0.21	125(79.6)	28(17.9)	4(2.5)	0.11
>Mean age										
Not identified										
Gender										
Male	177(51.8)	29(16.4)	91(51.4)	35(19.8)	22(12.4)	0.59	130(73.5)	42(23.7)	5(2.8)	0.98
Female	165(48.2)	26(15.8)	86(52.1)	39(23.6)	14(8.5)		122(73.9)	38(23)	5(3.1)	
Mean tumor size (mm)										
≤Mean	236(67.6)	31(13.2)	125(53.5)	56(23.5)	24(9.8)		169(71.6)	59(25)	8(3.4)	
>Mean	112(32.4)	24(21.4)	54(48.2)	21(18.8)	13(11.6)	0.20	85(75.9)	24(21.4)	3(2.7)	0.76
Not identified										
Differentiation										
Well	121(35)	18(14.9)	64(52.9)	26(21.5)	13(10.7)		95(78.5)	20(16.5)	6(5)	
Moderate	192(55.5)	32(16.7)	100(52.1)	40(20.8)	20(10.4)	0.82	139(72.4)	50(26)	3(1.6)	0.02
Poor	33(9.5)	5(15.2)	14(42.4)	11(33.3)	3(9.1)		19(57.6)	13(39.4)	1(3)	
Not identified										
Primary tumor (PT) stage										
pT1	52(17.6)	9(17.3)	31(59.7)	10(19.2)	2(3.8)		43(82.7)	8(15.4)	1(1.9)	
pT2	133(44.9)	21(15.8)	73(54.9)	26(19.5)	13(9.8)	0.29	104(78.2)	27(20.3)	2(1.5)	<0.001
pT3	100(33.8)	21(21)	46(46)	17(17)	16(16)		73(73)	23(23)	4(4)	
PT4	11(3.7)	1(9.1)	6(54.5)	1(9.1)	3(27.3)		8(72.7)	0(0)	3(27.3)	
Not identified										
Vascular invasion (VI)										
Present	59(17.2)	14(23.7)	18(30.5)	18(30.5)	9(15.3)		36(61)	21(35.6)	2(3.4)	
Absent	284(82.8)	40(14.1)	160(56.3)	57(20.1)	27(9.5)	0.13	215(75.7)	61(21.5)	8(2.8)	0.078
Not identified										
Lymph node invasion (LNI)										
Involved	118(34.3)	25(21.2)	48(40.7)	27(22.8)	18(15.3)	0.009	79(66.9)	33(28)	6(5.1)	0.007
None	226(65.7)	29(12.8)	130(57.5)	49(21.7)	18(8)		173(76.5)	49(21.7)	4(1.8)	
Not identified										

Neural invasion (NI)										
Involved	67(19.5)	12(17.9)	28(41.8)	19(28.4)	8(11.9)	<i>0.30</i>	42(62.7)	24(35.8)	1(1.5)	<i>0.41</i>
None	276(80.5)	42(15.3)	150(54.3)	56(20.3)	28(10.1)		209(75.7)	58(21)	9(3.3)	
Not identified										
Distant metastasis										
Present	67(28.5)	9(13.4)	30(44.8)	20(29.9)	8(11.9)	<i>0.51</i>	45(67.2)	18(26.8)	4(6)	<i>0.46</i>
Absent	168(71.5)	19(11.3)	91(54.2)	36(21.4)	22(13.1)		118(70.2)	44(26.2)	6(3.6)	
Not identified										
Tumor recurrence										
Yes	72(30.6)	9(12.5)	33(45.9)	22(30.5)	8(11.1)	<i>0.33</i>	46(64)	22(30.5)	4(5.5)	<i>0.48</i>
No	163(69.4)	19(11.7)	88(54)	34(20.8)	22(13.5)		117(71.8)	40(24.5)	6(3.7)	
Not identified										
<i>P value</i> ; Pearson's χ^2 test										
<i>H-score</i> histological score										
Values in bold are statistically significant										

Table 2. Cytoplasmic expression of DCLK1s in colorectal cancer (CRC) and adjacent normal tissue samples			
Scoring system	CRC samples N (%)	Adjacent normal tissue samples N (%)	<i>P-value</i>
Intensity of staining			
Negative (0)	55(15.8)	28(54.9)	<0.001
Weak (+1)	180(51.7)	20(39.2)	
Moderate (+2)	77(22.2)	3(5.9)	
Strong (+3)	36(10.3)	0(0)	
Percentage of positive tumor cells			
<25%	83(23.9)	33(64.7)	<0.001
25–50%	29(8.3)	2(3.9)	
51- 75%	56(16.1)	4(7.8)	
> 75%	180(51.7)	12(23.6)	
H-score (3 groups)			
Low (0-100)	255(73.3)	48(94.1)	<0.001
Moderate (101-200)	83(23.8)	3(5.9)	
High (201-300)	10(2.9)	0(0)	
Total	348	51	
<i>H-score</i> histological score			
<i>P value</i> is based on Mann–Whitney <i>U</i> test			
Values in bold are statistically significant			

Table 3. Univariate and multivariate Cox regression analyses of potential prognostic factor for disease-specific survival (DSS) in patients with colorectal cancer (CRC)

Covariate	Univariate analysis		Multivariate analysis	
	HR (95% CI)	<i>P</i> -value	HR (95% CI)	<i>P</i> -value
Cytoplasmic DCLK1s expression		0.04		0.14
High versus Low	1.61(0.97- 2.68)	0.06	1.22(0.67-2.22)	0.51
High versus Moderate	2.58(1.023- 6.52)	0.04	2.70(0.98-7.38)	0.04
Age	1.65(0.79-2.34)	0.04	1.73(1.007-1.99)	0.02
Tumor differentiation		0.01		0.94
Moderate versus well	1.46(0.86- 2.49)	0.15	1.34(0.75-2.38)	0.23
Poor versus well	2.99(1.47- 6.11)	0.003	2.31(0.85-6.31)	0.31
Primary tumor (PT) stage		0.02		0.22
pT2 versus pT1	1.15(0.49- 2.67)	0.74	1.09(0.46-2.57)	0.83
pT3 versus pT1	2.38(1.04- 5.45)	0.04	1.85(0.78-4.38)	0.15
pT4 versus pT1	1.08(0.22- 5.24)	0.91	0.94(0.17-4.98)	0.94
Vascular invasion (VI)	1.84(1.08-3.11)	0.02	-	-
Neural invasion (NI)	0.57(0.34-0.94)	0.03	-	-
Lymph node invasion (LNI)	0.45(0.28-0.71)	0.001	-	-
Distant metastasis	0.17(0.10-0.28)	<0.001	-	-
Tumor recurrence	0.18(0.11-0.30)	<0.001	-	-
<i>HR</i> hazard ratio, <i>CI</i> confidence interval				
The variables with <i>P</i> value less than 0.05 and HR more than 1.0 were included in multivariable analyses				
Values in bold are statistically significant				

Figures

Figure 1

Different localization pattern of DCLK1-S expression in CRC tissues by IHC staining: A) cytoplasmic expression, B) membranous, and C) nucleus expression of DCLK1-S. (All images were taken at 400 × magnification).

Figure 2

Immunohistochemical staining of DCLK1-S in CRC tissues and negative controls. (A) Negative, (B) low, (C) moderate, and (D) high expression of DCLK1-S in CRC tissue. Replacement of the primary antibodies by (E) pre-immune purified rabbit IgG (isotype control) and (F) Tris Buffer Saline, and (G) primary antibody saturated with immunizing peptide, as the negative reagent controls. (H) Negative expression of DCLK1-S in adjacent normal tissue (All images were taken at 200x magnification).

Figure 3

Box plot analysis of DCLK1-S expression levels in CRC specimens versus adjacent normal tissues. The results of Mann–Whitney U test showed that there is a statistically significant differences in the median expression levels of DCLK1-S among CRC and adjacent normal tissues ($P < 0.001$). Based on standard definitions, each box-plot demonstrated the median (bold line) and interquartile lines (box) outliers (circle), and extreme observations (star).

Figure 4

Kaplan-Meier curves for disease-specific survival (DSS) and progression-free survival (PFS) according to the expression levels of DCLK1-S in CRC. The findings represented (A) there is a significant differences between DSS and the patients with high, moderate and low expression of DCLK1-S ($P = 0.034$) and (B) there was no significant differences between PFS and the patients with high, moderate and low DCLK1-S expression ($P=0.175$).

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