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Original Article

Divergent expression of DCLK1 in gastrointestinal neuroendocrine tumors and primary hepatic, gallbladder, and pancreatic neuroendocrine tumors

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Abstract: Doublecortin-like kinase 1 (DCLK1) is reported to be a negative prognostic marker in colorectal cancer and is involved in tumorigenesis and progression through several miRNA pathways. In this study, We analyzed its expression in neuroendocrine tumor (NET) and explored its relation with survival outcome. 122 patients were enrolled in the study, including 60 cases of GI-NETs, 24 cases of primary hepatic NETs (PHNETs), 16 cases of gallbladder NETs (GBNETs) and 22 cases of pancreatic NETs (pNETs). IHC was performed for DCLK1 on tumor tissue. All patients underwent a baseline visit, histologic determination, and a follow-up for survival. In the 60 cases of GI-NETs, DCLK1 showed diffuse cytoplasmic expression. The positive rates of DCLK1, Syn and CgA were 100% (60/60), 100% (60/60) and 36.7% (22/60), respectively. However, DCLK1 showed negative staining in all of the 62 cases of PHNETs, GBNETs, and pNETs. The mean score of DCLK1, Syn, and CgA were (5.77±2.012), (5.13±2.078) and (2.68±2.797), respectively. DCLK1 was correlated with primary site ($P<0.001$) and Syn expression ($P = 0.045$). Additionally, in GI-NETs, we found that DCLK1 expression was associated with worse OS (log-rank = 5.212, $P = 0.022$). The divergent expression of DCLK1 in NETs suggests different functional roles of DCLK1 in different locations of NET within the digestive system. However, with the limited number of tumor samples, its outcome prediciton still needs further investigation. DCLK1 expression may aid in the diagnosis and prognosis of GI-NETs.

Keywords: Doublecortin-like kinase 1, neuroendocrine tumor, carcinoid, prognosis

Introduction

Neuroendocrine tumors (NETs), which arise from diffuse neuroendocrine cells, are widely distributed in the body. They most commonly originate in the lungs, pancreas, and gastrointestinal tract, but also less often other organs like breast, liver, bladder and biliary tree [1, 2]. Diagnosis and treatments of these tumors have been aided by pathologic, genetic and molecular advances; however, the survival of these patients remains largely unchanged [3].

Doublecortin-like kinase 1 (DCLK1) is a microtubule-associated protein with two doublecortin domains in the N-terminus that regulates osteoblast function, neuronal migration, and differentiation [4]. It was also demonstrated to be a cancer stem cell (CSC) marker in gastrointestinal cancers [5-7] and acted as a functional protein contributing to tumorigenesis and progression by several miRNA pathways [8, 9]. Apart from this, increased expression of DCLK1 has also been observed in hepatocellular carcinoma, pancreatic tumor, colorectal cancer and esophageal adenocarcinoma [10-13]. In particular, its overexpression has been claimed to be positively related to lymphatic metastasis, TNM stage, and is a poor prognostic factor in colorectal cancer [14, 15].

Recent studies indicate that DCLK1 shows diffuse cytoplasmic expression in rectal neuroendocrine tumors graded G1 and co-expresses with NANOG, a stemness marker [16]. In invasive breast cancer with neuroendocrine differentiation (IBC-NED), DCLK1 is associated with better overall survival (OS) and disease free survival (DFS) [17]. However, apart from that, no additional information is currently available about DCLK1 in NETs. It is not clear whether DCLK1 can be detected in rectal neuroendo-

Table 1. Pathologic grading in different primary locations of NETs

Primary tumor location	Pathologic grading			
	G1	G2	G3	n
Liver	2	4	18	24
Gallbladder	2	2	12	16
Pancreas	2	8	12	22
Stomach	0	0	32	32
Duodenum	6	4	8	18
Sigmoid colon	0	0	2	2
Rectum	8	0	0	8
Total	20	18	84	122

crine tumors graded G2 or G3, and other gastrointestinal NETs. Historically, the heterogeneous nature of NETs has been inherently complicated, and it was of interest to explore DCLK1 expression in different locations of NET.

The aim of this study was to evaluate the expression pattern of DCLK1 in digestive system NETs and its correlation with clinical features, as well as with survival outcome.

Methods and methods

Patient data

The histologic files of the involved institution (Chinese PLA general hospital) were searched for NET from January 2009 through December 2011. The patients who underwent tumor resection for gastro-intestinal, primary hepatic, gallbladder, and pancreatic NETs were enrolled. All the specimens were fixed in 10% formaldehyde solution and embedded in paraffin for histopathologic analysis. The 4 micron slides were stained with H&E and reviewed by two of the authors. Patient details and clinical information were retrieved from the medical records including age, sex, and tumor size.

For the outcome data, overall survival (OS) was defined as the time interval from the date of surgery to the date of cancer related death. Disease-free survival (DFS) was defined as the duration from the date of surgery to the first detection of cancer specific relapse or death. The last date of follow-ups was in April 2016 and the median follow-up period was 32.5 months (7-72 months). The study was approved by Chinese Ministry of Health and the Ethics Committee of the Chinese PLA General Hospital, Beijing, China.

Immunohistochemical analysis

DCLK1 and neuroendocrine markers (synaptophysin (Syn) and chromogranin (CgA)) in samples were assessed by an immunohistochemical method. After routine deparaffinization and rehydration, 4-μm slides went through epitope retrieval in citrate buffer (PH = 6.0) at 120°C for 4 minutes using pressure cooker. Blocking the endogenous peroxidase activity was performed in 3% hydrogen peroxide at room temperature for 15 minutes prior to the addition of primary antibodies. For blocking nonspecific binding sites, slides were exposed to goat serum at room temperature for 30 minutes. Sections were then incubated with anti-DCLK1 (1:700, ab109029; Abcam, Cambridge, MA) overnight at 4°C thermostat. Subsequent to being washed by PBS and distilled water, the sections were incubated with anti-rabbit secondary antibody (PV-6001) at 37°C for 30 minutes. Slides were then developed with diaminobenzidine (DAB, 1:20, ZLI-9017) to visualize the antibody reaction. For Syn and CgA staining, the anti-Syn (1:500; ab32127; Abcam) and anti-CgA (1:500; ab15160; Abcam) were used. Slides were examined with a Nikon 80i microscope and DXM1200C camera for brightfield microscopy.

The slides were scored for the intensity of staining in the nucleus, cytoplasm, or membrane according to different antibodies by two of the pathologists blinded to the clinical information and the staining results of other markers. For DCLK1 staining, the reactivity assessed was cytoplasmic. DCLK1 was assessed for both intensity and proportion of positively stained cells. A score of 0, 1, 2 and 3 was used to evaluate the staining intensity of nonreactive, weak, moderate and strong. Similarly, the proportion of stained cells was also scored on a scale of 0-4 (0 = no detectable staining, 1 = 1-25% positive cells, 2 = 26-50% positive cells, 3 = 51-75% positive cells and 4 = over 75% positive cells) [14, 17]. The two scores were combined to calculate the staining result, which generated scores of 0, 1, 2, 3, 4, 6, 9, and 12. Immunoscore of 0-3 was regarded as negative and >3 as positive.

Statistical analysis

Data were analyzed with SPSS version 22.0 for Mac (SPSS Inc, Chicago, IL), and significance

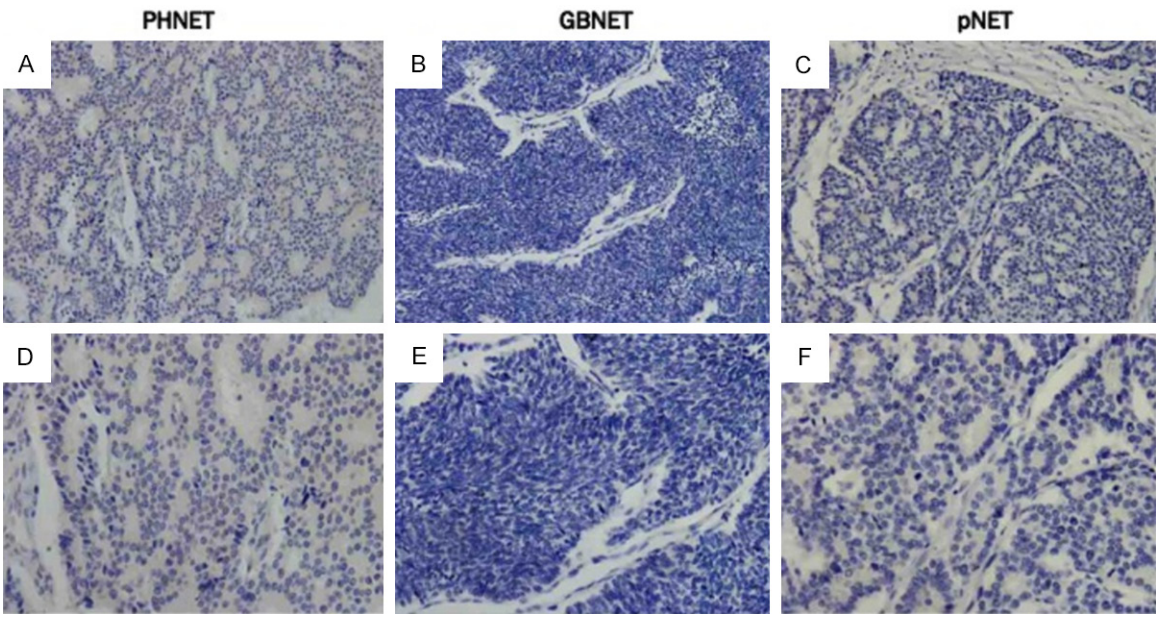

Figure 1. Expression of DCLK1 in NET tissue specimens. Note the negative expression of DCLK1 in PHNET (A, D), GBNET (B, E) and pNET (C, F) patients.

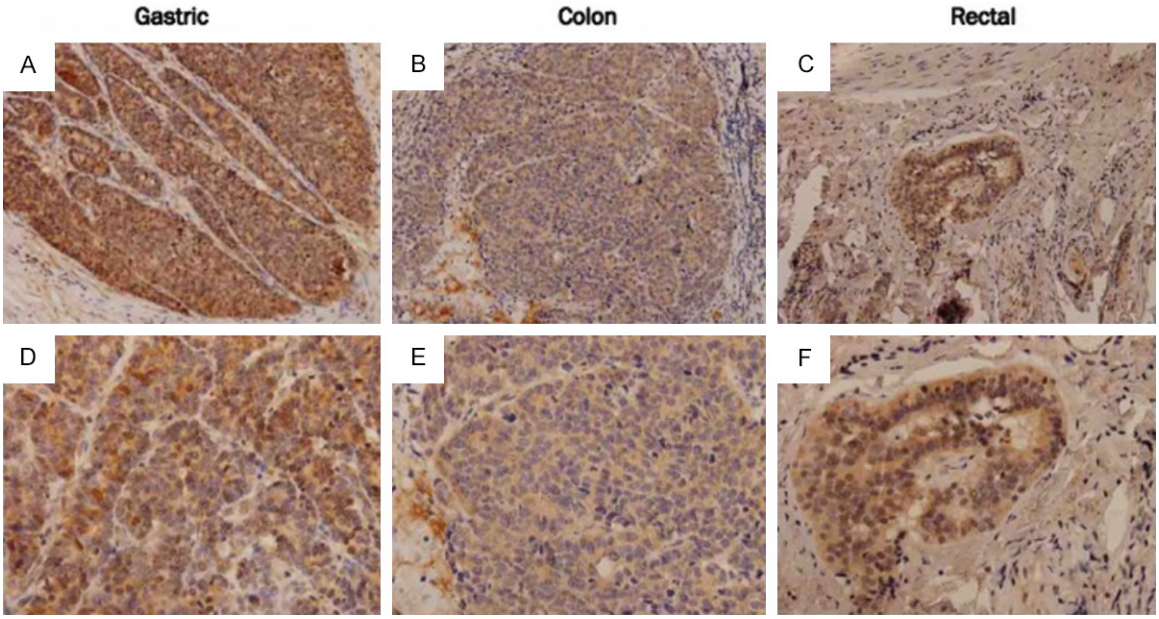

Figure 2. Representative immunohistochemical staining of DCLK1. Note the high cytoplasmic immunoreactivity of DCLK1 in tumor cells but not in surrounding stroma. DCLK1 in gastric NET (A, D), colonic NET (B, E) and rectal NET (C, F) patients.

was set as $P<0.05$. byIndependent-sample T test, one way analysis of variance (ANOVA) and Mann-Whitney U test were used to compare the groups of patients. Pearson correlation was to measure the linear correlation between two variables. The median DFS and OS were measured from inception and estimated by using the Kaplan-Meier analysis and Cox regression.

Results

Expression of DCLK1 in GI-NETs

A total of 122 cases were included in this study. The mean age was 52.2±1.5 years (range 24-81 years). The mean tumor size was 5.7±4.9 cm (range 0.5-21.0 cm). 20 (16.4%)

Table 2. Correlation of DCLK1 expression with clinicopathologic features

		DCLK1				Score		
		Negative	Positive	Total	*P* value	Mean	SD	*P* value
Sex	Male	40	39	79	0.71	2.29	2.813	0.279
	Female	22	21	43		3.48	3.121	
Grade	1	8	12	20	0.326	4.50	3.381	0.306
	2	12	6	18		2.00	2.449	
	3	42	42	84		3.26	2.980	
pN	Negative	38	42	80	0.990	3.26	2.690	0.649
	Positive	20	22	42		2.68	3.014	
Site	GI	0	60	60	<0.001			
	Others	62	0	62				
Syn	Negative	4	0	4	0.045	1.00	1.414	0.102
	Positive	58	60	118		3.14	3.071	
CgA	Negative	34	38	72	0.340	3.31	3.078	0.701
	Positive	28	22	50		2.72	3.035	
CD56	Negative					4.09	3.506	0.335
	Positive					3.84	2.930	
Tumor size	Mean	7.15	5.29	0.402				
	SD	5.971	2.952					

Table 3. Association of DCLK1 expression with biomarkers in GI NETs

		Score			
		N.	Mean	SD	*P* value
Sex	Male	46	5.71	2.215	0.833
	Female	14	5.78	1.999	
Grade	1	12	6.00	2.280	0.822
	2	6	5.00	1.414	
	3	42	5.62	1.962	
pN	Negative	36	5.49	1.748	0.755
	Positive	24	5.73	2.207	
Site	Gastric	32	6.13	1.893	0.513
	Intestinal	28	5.36	2.134	
Syn	Negative	0	0	0	0.367
	Positive	60	5.77	2.012	
CgA	Negative	38	5.84	1.922	0.494
	Positive	22	5.64	2.248	
CD56	Negative	14	6.29	2.215	0.832
	Positive	46	5.61	1.971	

were grade I, 18 (15.5%) were grade II and 84 (68.8%) were grade III (**Table 1**). There were 24 cases of primary hepatic NETs (PHNETs), 16 cases of gallbladder NETs (GBNETs), 22 cases of pancreatic NETs (p-NETs), 32 cases of gastric NETs (g-NETs), 18 cases of duodenal NETs (d-NETs), 8 cases of rectal NETs (r-NETs) and 2 cases of NET of sigmoid colon.

Overall, 60 (49%) were DCLK1 positive and 62 (51%) were DCLK1 negative. Representative staining is shown in **Figures 1** and **2**. In the 60 cases of GI-NETs, DCLK1 showed diffuse cytoplasmic expression. The positive rates of DCLK1, Syn and CgA were 100% (60/60), 100% (60/60) and 36.7% (22/60), respectively. However, DCLK1 showed negative staining in all of the 62 cases of PHNETs, GBNETs, and p-NETs. The positive rates of DCLK1, Syn and CgA were 0% (0/62), 93.5% (58/62), and 45.1% (28/62), respectively. The mean score of DCLK1, Syn and CgA were (7.70±2.46), (7.03±3.02) and (3.33±4.04), respectively. There were no significant differences in the scores between DCLK1 and Syn, but the two scores were significantly higher than that of CgA (P<0.05).

Correlation with clinicopathologic features and biomarkers

DCLK1 expression was correlated only with primary site (P<0.001), but not sex, tumor size, grade, and pN stage (**Table 2**). Apart from this, DCLK1 was also related to the expression of Syn (P = 0.045). There was no significant correlation with CgA and CD56.

Table 4. Pearson analysis of DCLK1 and Syn or CgA expression

Group	Mean	SD	Pearson	*P* value
DCLK1 staining	5.77	2.012		
Compared with Syn staining	5.13	2.078	0.026	0.892
Compared with CgA staining	2.68	2.797	0.148	0.435
Compared with CD56 staining	4.32	2.713	0.213	0.259

Relationship with clinicopathologic features and biomarkers in GI-NETs

In GI-NETs, no significant differences (P>0.05) were detected among GI-NET patients stratified by sex, grade, pN stage, and primary tumor site (**Table 3**). Regarding biomarkers, DCLK1 expression was not associated with the absence of Syn, CgA, and CD56. Further, Pearson analysis also did not show any correlation between DCLK1 and Syn, CgA, or CD56 expression (**Table 4**; **Figure 3**).

Relationship with outcome

Follow-up data were available for 122 cases with a mean follow-up of 32.5 months (range 7-72 months). In the 60 GI-NET patients, 46 (76%) had cancer-related death. The overall 1-year survival rate, 3-year survival rate, and 5-year survival rate were 86.7%, 43.3% and 23.3%, respectively.

Kaplan-Meier analysis demonstrated a significant impact of certain clinicopathologic prognostic factors such as DCLK1 staining level, grade, primary tumor site, tumor size, distant metastases, vascular invasion, and lymph nodes on overall survival (P<0.001, 0.023, <0.001, <0.001, <0.001, 0.009, and <0.001, respectively). No significant relations were found between OS and other clinicopathologic factors (**Table 5**).

We also found that DCLK1 was associated with worse OS (log-rank = 5.212, P = 0.022) but not with DFS (log-rank = 2.170, P = 0.141) in GI-NET patients (**Figure 4**). Cox regression analysis showed the prognostic impact on OS of GI-NETs was independent of tumor size, pN stage, and distant metastases (**Table 6**). DCLK1 was not an independent predictor for GI-NET patients. Subgroup analysis found that DCLK1 did function as a strong predictive biomarker in G-NET patient and intestinal tract NETs patient groups. However, it did not behave as a predictive biomarker in either of these groups (log-rank = 3.715. P = 0.054 and log-rank = 3.311, P = 0.069, **Figure 5**).

Discussion

In this study, we evaluate the expression pattern and assess its value in the prognosis of GINETs. We found that DCLK1 showed completely different expression patterns in involved GINETs and PHNETs, GBNETs, and p-NETs. The positive expression rate of DCLK1 in GI-NETs was 100% (60/60), which was much higher than in the PHNETs, GBNETs, and p-NETs (0/61) (P<0.001). Previous studies also have confirmed the strong positive expression of DCLK1 in rectal NETs [16], but the tumor grade and the number of tumor samples were limited. DCLK1 expression was not associated with sex, age, tumor grading and primary tumor site. However, we found that its expression was probably higher in patients with distant metastases. The pathologic diagnosis of NETs has to be established before arriving at a diagnosis in a specific location [18]. For biopsy-proven NETs of unknown primary, tumor-directed localizing studies such as multiphasic CT or MRI, somatostatin receptor scintigraphy, ultrasound, EUS, FDG-PET/CT scan, or EGD and/or colonscopy are needed to discover the primary site of tumor [19]. Since DCLK1 is highly expressed and found in GINETs, it may be used as a marker for the pathologic diagnosis and a sensitive marker in the differential diagnosis of digestive system NETs.

Several studies have demonstrated DCLK1 staining levels were elevated in several solid tumors. The positive rate has been reported to be 65% (88/135) in invasive breast cancers with neuroendocrine differentiation (IBC-NED) [17]. Ikezono reported that DCLK1 protein was observed in all the involved rectal neuroendocrine specimens [16]. Doublecortin-like kinase 1 (DCLK1, formerly known as DCAMKL-1) gene is located on the human chromosome 13q13.3 [20]. The encoded protein has two doublecortin (DCX) domains in the N-terminus for binding microtubules and regulation of microtubule polymerization [21] and a serine/threonine protein kinase domain in the C-terminus. The C-terminal domain resembles calcium/calmod-

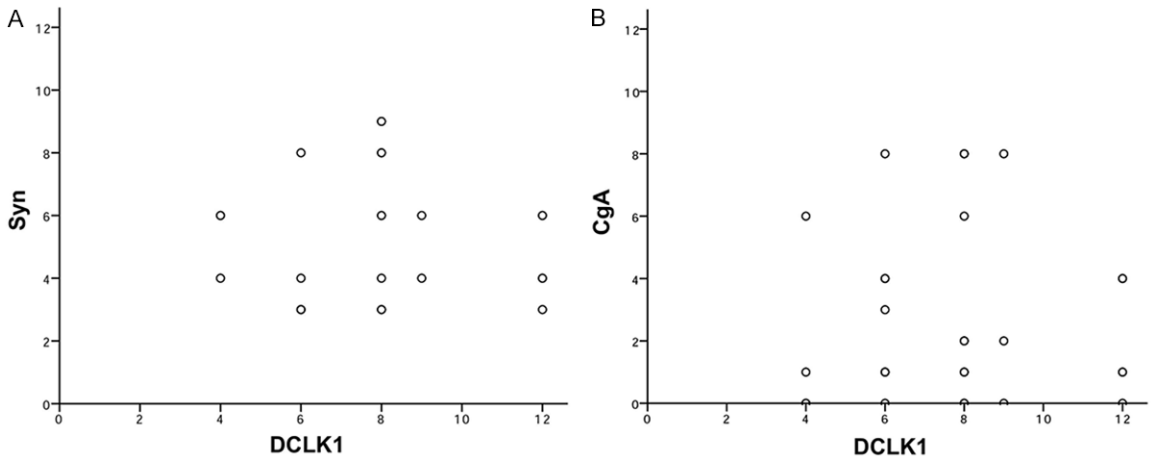

Figure 3. Pearson analysis comparing DCLK1 and Syn or CgA expression.

Table 5. Univariate analyses of factors associated with long-term survival in GI-NETs

Variable	Log-rank	Overall survival (*P*)
G-NETs vs intestinal tract NETs	11.932	<0.001
Grade(1 vs 2 vs 3)	4.278	0.023
Tumor size (≤5 cm vs >5 cm)	12.514	<0.001
Lymph nodes (positive vs negative)	11.765	<0.001
DCLK1 level (++ vs +++)		
Vascular invasion (positive vs negative)	6.211	0.009
Distant metastases (positive vs negative)	10.911	<0.001
Nerve invasion (positive vs negative)	7.645	0.178
Sex (male vs female)	0.145	0.892

ulin-dependent protein kinases II, but it lacks a canonical calmodulin-binding site [22]. The expression of DCLK1 in organs varies with age. In fetal tissues, DCLK1 is detected in brain, liver and lungs but not in kidneys. In adult tissues, DCLK1 is mainly found in the brain, heart, liver, spleen, small intestine and colon.

DCLK1 gene transcription is very complex and can produce different protein domains by alternative splicing and different promoter transcription [23]. More than nine different splice variants have been identified in the DCLK1 gene, including full-length isoforms enriched in arginine residues, isoforms of microtubule anchorage domain, and isotypes of kinase domain. One known splice variant is DCLK1-shortAwhichcanencodecalcium/calmodulin-dependent kinase (CaMK)-like proteins with different C-terminal ends. In addition, DCLK1-short A have two similar splice variants: DCLK1-short B and DCLK1-short C. They control different neuronal processes through different CaMKs. DCLK1-long, now refered to as DCLK1, has the activity of microtubule binding and polymerization. In adult brain, DCLK1 protein can regulate osteoblast function, neuronal migration, and differentiation. However, the specific biologic function of DCLK1 has remained unknown. Considering the different 3D structures, different isoforms are expected that differ in their biologic interactions and activities [24].

The heterogeneous nature of NETs is inherently complicated. GEP-NETs derive from cells of the diffuse neuroendocrine system (DNES) and hence are endodermal in origin. Hormonal substances of the endocrine cells are stored in the large dense core vesicle (LDCV) and the synaptic-like microvesicle (SLMV) [25]. Nonetheless, the endocrine cells of DNES are highly heterogeneous and consist of 14 different types of cells, resulting in GI-NENs and primary hepatic, gallbladder, and pancreatic NETs having different classification biochemically and histologically [3]. Without specialized DNES cells, the origin of PHNETs still has been in the arguments. It has been hypothesized that they derived from endocrine cells of intrahepatic cholangiocytes, local multipotent liver stem cells, and migration of the pancreatic or adrenal tissues [26].

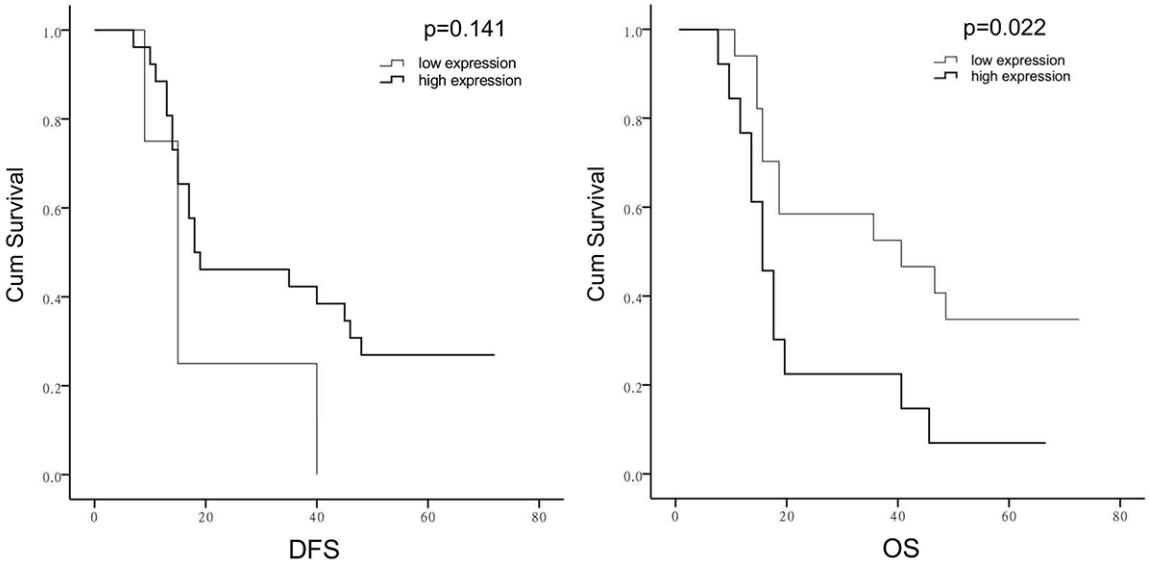

Figure 4. Kaplan-Meier analysis of DFS and OS in GI-NETs according to DCLK1 expression in GI NETs.

Table 6. Multivariate analysis of factors that might affect long-term survival in GI-NETs

Variable	Overall survival		
	HR	95% CI	*P*
Primary tumor site	1.081	0.303-3.857	0.139
Grade	0.238	0.057-1.002	0.078
Tumor size	1.186	1.074-2.182	<0.001
Lymph nodes	3.94	1.432-10.814	0.011
DCLK1 level	0.513	0.186-1.413	0.197
Vascular invasion	0.736	0.286-.893	0.542
Distant metastases	1.062	0.918-1.418	<0.001

In the present study, we observed the positive expression of DCLK1 in GI-NETs and negative expression in primary hepatic, gallbladder, and pancreatic NETs. The completely opposite expression pattern may be correlated with the complexity of the DCLK-gene and the heterogeneous nature of NETs to some extent. Furthermore, in a study by Hironori et al. DCLK1 was clearly expressed in a human pancreatic neuroendocrine tumor cell line and human neuroblastoma cell line by western blotting [27]. But, the specific data and mechanism were not described.

Previous studies looking at elevated DCLK1 expression as a prognostic factor have shown conflicting results. In gastrointestinal and pancreatic tumors, accumulating evidence suggests that DCLK1 is a marker of normal and cancer stem cells [5, 7, 28]. The function of DCLK1 is not fully understood; but we know that DCLK1 plays an essential role in cancer initiation and inducing epithelial-mesenchymal transition (EMT), which in turn supports the stemness nature of DCLK1+ cells. Regarding the mechanism of the DCLK1-induced EMT, involvement of specific microRNA-dependent pathways and dysregulation of ZEB1, ZEB2, c-MYC, KRAS, and Notch-1 expression was observed in pancreatic and colon cancers [8, 29, 30]. Furthermore, in the normal gastric stem cell niche, ablation of DCLK1+ cells or blockade of nerve growth factor (NGF) signaling inhibited epithelial proliferation and tumorigenesis in an acetylcholine (ACh) muscarinic receptor-3 (M3R)-dependent manner, in part through suppression of yes-associated protein (YAP) function [31]. Otherwise, in a large cohort of breast cancer, DCLK1 had a favorable correlation with a lower grade, absence of lymphovascular invasion, fibrotic foci, necrosis, lower pN stage, as well as better outcome [17]. DCLK1 was more frequent in invasive breast cancers with neuroendocrine differentiation (IBC-NED) and was found to be a good prognostic factor regardless of the NED expression pattern. In the present study, although increased DCLK1 staining levels were revealed to be correlated with mortality during follow-up, they were not identified as an independent predictor for identifying OS of GENET patients.

In addition, in the only case of primary neuroendocrine tumor of the breast (NETB), DCLK1

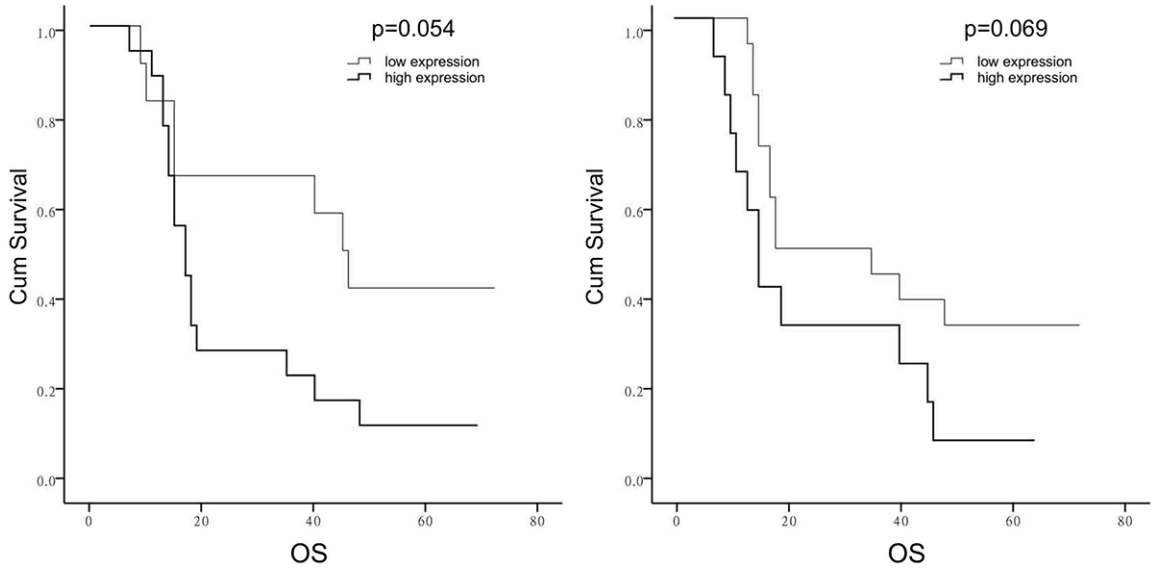

Figure 5. Kaplan-Meier analysis of OS in G-NET patients and intestinal tract NET patient groups.

showed isolated/scattered expression, and sometimes could be recognized at the surface of tumor cells. NETB is a heterogeneous group of rare tumors, the origin of which has been argued. Gubilla et al. reported that NECB cancer cells derived from argyrophilic cells of neural crest origin that migrated to the mammary ducts [32]. Maluf et al. insist on the theory that NECB derive from neoplastic stem cells which have the ability to differentiate into both epithelial and endocrine lines [33, 34]. Kawasaki et al. demonstrated the existence of extensively-distributed NE cells in the background tissues of NECBs using immunohistochemical examination [35]. They speculated that mammary NE cells are likely to be the result of a hyperplastic process of differentiated and/or metaplastic neuroendocrine cells. DCLK1 could have a biologic role in NETBs, but the specific mechanisms have yet to be sufficiently investigated and established.

To conclude, this study provides original findings and novel s regarding the expression of the CSC marker, DCLK1, in NETs originating in different locations. DCLK1 may be a sensitive marker in the differential diagnosis of digestive system NETs. However, further studies are required to determine the role of DCLK1 in NETs more precisely.

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Disclosure of conflict of interest

None.

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