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ORIGINAL ARTICLE

A new insight into cancer stem cell markers: Could local and circulating cancer stem cell markers correlate in colorectal cancer?

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Abstract Cancer stem cell (CSC) markers could serve as potential prognostic procedure. This study is aimed to investigate the local expression of doublecortin-like kinase 1 (DCLK1) and Lgr5 in colorectal cancer tissues (CRC) at both protein and messenger RNA (mRNA) level, followed by providing a comparison of the local and circulating expression pattern of these markers, based on our present and previous study. The mRNA expression level of DCLK1 and Lgr5 was evaluated using comparative real-time PCR method applying 58 fresh tumor tissues and their correspondent normal margins. Immunohistochemistry was applied to analyze the protein expression level of DCLK1 and Lgr5 in paraffin-embedded CRC tissues. The correlation of DCLK1 and Lgr5 expression pattern with clinicopathological characteristics was assessed. A higher mRNA expression level of DCLK1 (3.28-fold change, $p<0.001$) and Lgr5 (2.29-fold change, $p<0.001$) was observed in CRC fresh tissues compared to the normal adjacent margins, and the expression level was higher in patients with higher grade and stages of disease and patients who underwent neoadjuvant chemoradiotherapy (CRT). The protein expression level of DCLK1 and Lgr5 was also increased significantly in tumor tissues compared to normal colon tissues which were positively correlated to tumor stage and grade and neoadjuvant CRT. Taken together, the results of protein analysis were in accordance with mRNA assessment. The local expression pattern of DCLK1 and Lgr5 was also in accordance with their expression level in circulation. However, some minor inconsistencies were observed which may be attributed to several factors including the possible effect of CRT on CSC reprogramming.

Keywords Colorectal cancer · DCLK1 · Lgr5 · Local cancer stem cell · Circulating cancer stem cell

Introduction

A growing body of evidence supports the hypothesis that tumors are initiated from a subpopulation of tumor cells named "cancer stem cell" (CSC) [1–7]. Recently, CSC hypothesis has attracted much attention in both cancer management and therapy [8–11]. The acceptance of CSCs as the main players of

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tumor formation, survival, metastasis, resistance to treatment, and recurrence imposes a new perspective to profit CSCs. In this regard, reevaluation of tumor management focusing on CSCs as tumor root instead of tumor-differentiated cells as the tumor foliage should be taken into consideration [12].

Despite the significance of CSC hypothesis, application of CSC to clinical implications has faced many difficulties. Among them, similarity of CSC markers to normal stem cell markers [13] and the heterogeneity of CSC markers [14, 15] are considered as the most prominent drawbacks.

Doublecortin-like kinase (DCLK1) and Lgr5 are considered as the most potential colorectal CSC markers, and their role has been demonstrated in several studies [16–21]. Diagnostic and prognostic role of DCLK1 and Lgr5 has also been shown in blood circulation of colorectal cancer (CRC) patients, indicating traceability of circulating CSCs [22, 23]. The potentiality of DCLK1 as a colorectal CSC marker has become even more prominent following the study of Nakanishi et al. which have used lineage tracing to show that DCLK1 does not mark normal intestinal stem cells but instead marks intestinal tumor stem cells. Accordingly, it generates new hope in specific targeting of CSC without damaging normal stem cell pool [24]. They also showed that Lgr5 is co-expressed with DCLK1 on colorectal CSCs as well as normal intestinal stem cells.

However, a question might be raised regarding whether there is a difference in expression pattern of CSC markers in tumor tissue, known as local CSC markers (LCSC), and CSC markers in the peripheral blood (PB) of the patients, known as circulating CSC (CCSC) markers. In spite of CSC markers' diagnostic and prognostic significance, no study has been performed in order to compare the expression pattern of local CSC markers with the expression pattern of circulating CSC markers. Therefore, we have designed a connected set of studies concerning diagnostic and prognostic potential of DCLK1 and Lgr5 as highly proposed colorectal CSC markers. In the first part of the study, we investigated the DCLK1 and Lgr5 messenger RNA (mRNA) expression pattern in blood circulation of CRC patients [22]. In the current study, the local mRNA expression of DCLK1 and Lgr5 was evaluated in tumor tissues of the same CRC patients. Finally, the results of local expression of DCLK1 and Lgr5 from the current study were compared with our previous data regarding the expression level of these markers in circulation. Application of blood and tissue samples of the same patients made the results of the current study completely comparable with our former study. This comparison can be considered as a piece of CSC puzzle which may pave the way for future investigations and provide prerequisite information on the prognostic potentiality of DCLK1 and Lgr5 markers in colorectal cancer. To our knowledge, this is the first study comparing the local and circulating cancer stem cell markers' expression pattern.

Since proteins are the final product of the genes and mRNA expression does not necessarily accord with protein expression, comparison of mRNA and protein expression of the markers may also result in valuable information. Therefore, the protein expression of DCLK1 and Lgr5 was also examined in the paraffin-embedded sections of the same series of CRC patients using immunohistochemistry to enquire CSC markers' protein and mRNA conformity.

Material and methods

Patients and samples

Since the beginning of 2013, in a prospective study, the blood and fresh tissue samples of the CRC patients referred to Imam Khomeini Hospital of Tehran University of Medical Sciences were collected. The collection included 78 blood samples and 62 fresh tumor tissues. Fresh CRC tissue samples as well as paired normal margins were obtained immediately after surgery and kept on liquid nitrogen prior to be stored at −80 °C freezer. Paraffin-embedded tissue and correspondent H&E slides of the same patients were also acquired. Since the purpose of the study was to compare the circulating and local expression pattern of the markers, those samples in which their blood, fresh, or paraffin-embedded tissue were missing have been excluded from the study. Finally, a total of 58 patients in which their blood, fresh, and paraffin-embedded tissue were available were included in the study. The circulating mRNA expression of DCLK1 and Lgr5 in blood samples of CRC patients has been previously examined [22], while the local mRNA expression of these markers in tissue samples of CRC patients was investigated in the present study. Totally, 34 patients with colon and 24 patients with rectum cancer were enrolled in the study. Male patients comprised 53.5 % of the study population, versus 46.5 % of female patients. The median patients' age was 74.2 years, ranging from 28 to 86 years. Tumors were staged and graded according to TNM classification protocol [25]. Altogether, 11 patients with stages I and II, 28 patients with stage III, and 19 patients with stage IV of colorectal adenocarcinoma were classified.

Considering the positive effect of preoperative chemoradiotherapy (CRT) on tumor management and morbidity reduction in locally advanced tumors [26, 27], neoadjuvant CRT was applied for stages II to IV of the disease according to the protocol proposed by German rectal cancer study group [27]. The main neoadjuvant chemotherapy regimen included an intravenously infusion of a dose of 1000 mg/m^2/day of 5-fluorouracil (5-FU) throughout the first and fifth weeks of radiotherapy. Preoperative radiotherapy included the administration of a total dose of 50 GY which was given in 25 fractions through 5 weeks. A number of 33 qualified patients received preoperatively CRT treatment. The patients' clinical characteristics are shown in details in.

This study was performed under the supervision and approval of the Ethics Committee of clinical investigation of Tehran University of Medical Sciences, and written consents were obtained from all participant patients.

RNA extraction, cDNA synthesis, and primer design

A total of 0.5 g of resected tissue samples was preprocessed and homogenized using liquid nitrogen. Afterward, 200 μg of proteinase K was added to each homogenized sample and incubated at 37 °C for 1 h in order to digest unwanted proteins. Following RNA extraction using TRIzol reagent and DNase treatment of extracted RNA, complementary DNA (cDNA) was synthesized using Revert Aid First-Strand cDNA Synthesis Kit according to the company instructions (Thermo Scientific, USA). Real-time PCR was performed applying SYBR® Premix Ex Taw™ II (Takara, Japan). Beta-actin was selected as the reference gene of choice, and its primers were purchased from Qiagen Company (Hs_Actb_1_SG, QuantiTect Primer Assay QT00095431, Qiagen, USA). Lgr5 and DCLK1 primer sets were designed with AlleleID 6.0 software and were synthesized by Metabion Company (Germany) [22]. Their sequences are as follows: DCLK1 F: AGGGTCGTAA ACTGGTGGGA AAC, DCLK1 R: TGTCTGTATG GGCAAGATAT GGTAAAC and Lgr5 F: CTGAACTAAG AACACTGACT CTGAATG, Lgr5 R: CACTTGGAGA TTAGGTAACT GATTGC.

Real-time PCR

Real-time PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) for 1 cycle at 95 °C for 2 min followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Specificity of the products was approved by melting curve analysis, and positive and negative controls were included in each run. Data was analyzed according to comparative Ct method [28]. $2^{-\Delta Ct}$ where ΔCt=(CT of target gene−CT of internal control) was calculated in order to obtain mean ± SD of each sample mRNA expression for further statistical analysis. Considering the basic assumption of comparative Ct method which is the approximately equal amplification efficiency of target and reference genes, average efficiency of each gene was calculated using real-time PCR Miner system [29]. Comparable efficiency of β-actin (95.7), DCLK1 (94.8), and Lgr5 (96.6) approved the comparative Ct method for quantitative evaluation of DCLK1 and Lgr5 mRNA levels.

Tissue microarray

The tissue microarrays (TMA) were constructed as described in our previous studies [30–32]. To this aim, four different tumor regions were marked on each H&E slides, and the marked regions was transferred into their correspondent paraffin-embedded tissue blocks. Subsequently, from each marked region on the donor blocks, a microarray sample with the diameter of 0.6 mm were punctuated and precisely arrayed into a new recipient paraffin block using Tissue Arrayer Minicore (ALPHELYS, Plaisir, France). Totally, four copies of TMA block was constructed, each containing one sample from different regions of the same paraffin-embedded block. The mean scoring of the four cores was calculated as the final score.

Immunohistochemical staining

Immunohistochemistry was performed on a 4-μm tissue sections using rabbit polyclonal anti-DCLK1 (Abcam, UK, ab37994) and anti-Lgr5 (Abcam, UK, ab71225) as primary antibodies.

Briefly, following deparaffinization and then rehydration of slides, TMA sections were immersed in methanol containing 0.3 % hydrogen peroxide for 20 min to block possible endogenous peroxidase activity. Afterward, antigen retrieval process was performed using autoclaving the section in citrate buffer (pH=6) for 10 min. Sections were then incubated with primary antibodies with optimal dilutions which were found to be 1/200 for DCLK1 and 1/400 for Lgr5 for 2 h at room temperature. Sections were then incubated with secondary antibody (Envision System, Dako, Denmark) for 1 h at room temperature and after that were treated with 3,3′-diaminobenzidine (DAB, Dako) in order to visualize antigen/antibody reaction. Finally, counterstain was performed using hematoxylin (Dako), and after dehydration steps, the slides were mounted to be analyzed. The primary antibody was omitted from negative control slides and replaced with washing buffer.

Assessment of immunohistochemical staining

Immunostained tissue arrays were scored semiquantitatively by two specialist observers without knowledge of the clinical and pathologic parameters of patients, and in difficult cases, a consensus was achieved. TMA slides were initially scanned at low magnification to obtain a general impression of the overall distribution of the positive cells and then assessed semiquantitatively at higher magnifications, and final scores were given. The intensity of staining was assigned on a scale of 1 to 3 as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cells was also assessed semiquantitatively. The histochemical score (H-score) was obtained by multiplying intensity and percentage of positive cells, and a final score of 0 to 300 was given [33].

Statistical analysis

In order to evaluate the mRNA expression level of Lgr5 and DCLK1 in tumor tissues compared to normal adjacent margins, parametric tests including paired *t* test, one-way

ANOVA, and Pearson correlation coefficient test were used to assess significance of differences. Non-parametric tests such as chi-squared and Spearman's correlation coefficient test were used for univariant analysis of immunohistochemistry (IHC) results. Statistical analysis of data was performed using IBM SPSS Statistics 22 and a p value of <0.05 was considered statistically significant.

Results

DCLK1 and Lgr5 mRNA level was increased in CRC tissues

The mean and standard deviation (SD) of Lgr5 and DCLK1 mRNA expression level in fresh tumor tissues of CRC patients and adjacent margins were calculated using $2^{-\Delta Ct}$, and the related fold changes was obtained. For ease of calculation, decimals up to five digits have been removed. The mean of Lgr5 mRNA expression level was 983.3 (SD=265.6) in CRC tumor tissues and 428.5 (SD=186.7) in due margins (Fig. 1).

These data indicates a 2.29-fold change of Lgr5 mRNA expression in resected tumor tissues compared with normal adjacent margins that was statistically significant ($p<0.001$). In addition, Lgr5 expression level was significantly higher in patients at stages III and IV comparing to stages I and II ($p=0.02$). The significant higher expression of Lgr5 was also seen in poorly differentiated tumors ($p=0.024$). Moreover, the expression level was significantly higher in patients with the history of preoperative CRT compared to those lacking the neoadjuvant CRT history ($p=0.019$). There was no other significant correlation between the expression level of Lgr5 and other clinicopathologic findings such as age, sex, tumor location, and tumor size.

The mean expression level of DCLK1 was 165.4 (SD=66.3) in resected tumor tissues and 50.3 (SD=21.6) in adjacent margins (Fig. 1). As a result, a 3.28-fold change was seen in tumor area compared to related margins that was statistically significant ($p<0.001$). The expression level was also significantly higher in poorly differentiated tumors ($p=0.022$) and in patients at stages III and IV comparing to stages I and II ($p=0.015$). In addition, in preoperative CRT-treated patients, DCLK1 mRNA expression was significantly higher compared to those lacking the history of CRT ($p=0.01$). The correlation between DCLK1 and other clinicopathologic data was not statistically significant. The correlation of DCLK1 and Lgr5 mRNA expression with patients' clinicopathologic findings has been summarized in Table 1.

Pearson's correlation coefficient test was applied in order to evaluate the correlation between local DCLK1 and Lgr5 mRNA expression level as well as specify the coloration between the local and circulating mRNA expression level. The statistical analysis elucidated a positive correlation between the mRNA expression level of DCLK1 and Lgr5 in tumor tissues of CRC patients ($r=0.512$, $p=0.002$). Furthermore, by comparing the level of mRNA expression in tissue samples with mRNA expression in blood of the same patients from our previous study [22], it has been revealed that there is a significant correlation between the mRNA expression level of DCLK1 in CRC tissue samples (local) with its expression level in circulation ($r=0.602$, $p=0.001$). Similarly, the mRNA expression level of LGR5 in tissue samples was significantly correlated with circulating Lgr5 mRNA level ($r=0.481$, $p=0.002$).

In order to facilitate the comparison of mRNA level between tumor tissues and normal margins, Youden index was used to assign a cutoff value for each of two genes. In this regard, the calculated cutoff for Lgr5 and DCLK1 found to be 759 and 96, respectively.

DCLK1 and Lgr5 protein level was increased in CRC tissues

A number of 58 paraffin-embedded tissues from CRC patients were arrayed on TMA blocks and examined by immunohistochemistry. Since normal colon and rectum are known to express DCLK1 and Lgr5, these tissues were used as positive control. H-score method was used for assessment of expression, and the median H-score was chosen as cutoff value in order to classify the cases as high and low expressive. Consequently, an H-score of 20 was assigned as cutoff point for DCLK1, while an H-score of 30 was determined as the cutoff value of Lgr5. In this regard, DCLK1 expression was found to be high in 37 of 58 (63.7 %) tumor tissues (Fig. 2).

The overexpression of DCLK1 was significantly correlated with tumor stage indicating that 72.3 % of stages III and IV and 27.3 % of stages I and II of the CRC patients revealed high expression ($p=0.014$). In addition, a positive correlation was observed between DCLK1 overexpression and tumor grade as 83.3 % of poorly differentiated tumors versus 53.8 % of moderately differentiated and 37.5 % of well-differentiated tumors have shown high DCLK1 expression comparatively ($p=0.023$). Besides, comparison of tumors of patients undergoing neoadjuvant CRT with the patients lacking the history of CRT showed a significant overexpression of DCLK1 in the preoperative CRT-treated patient (78.8 %) compared to patient without CRT history (44 %) ($p=0.016$). No significant correlation was found between DCLK1 expression and other clinicopathologic parameters including sex, age, tumor location, and tumor size.

High Lgr5 expression level was also observed in 30 out of 58 (51.7 %) CRC samples (Fig. 2). Similarly, Lgr5 overexpression was significantly correlated with tumor stage indicating 65.9 % of patients with stages III and IV, and 27.2 % of patients with stages I and II of CRC have shown high Lgr5 expression ($p=0.045$). Likewise, poorly differentiated tumors

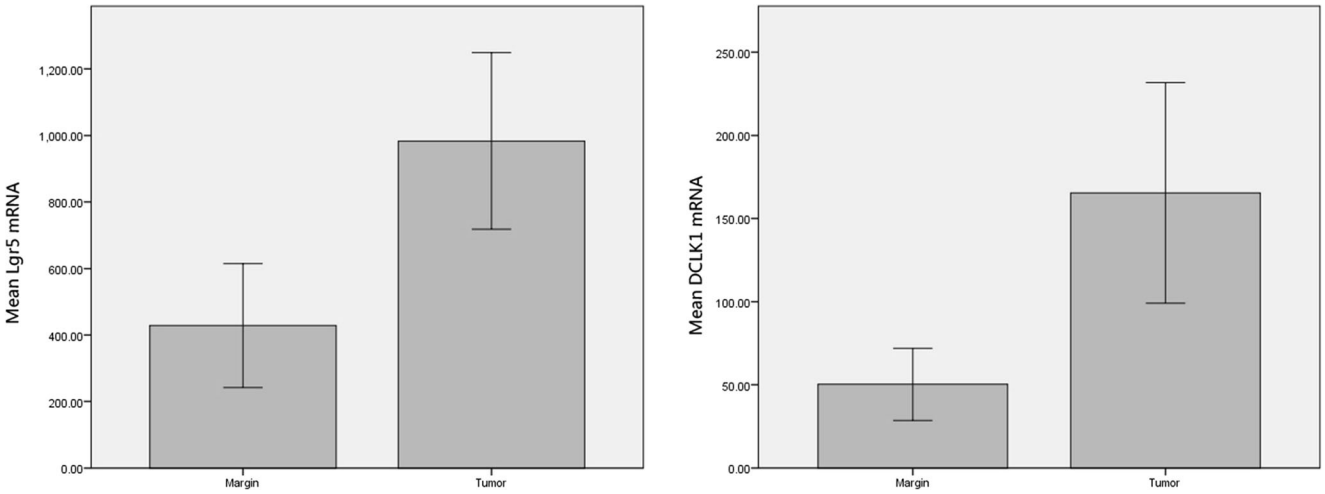

Fig. 1 qRT-PCR analysis of DCLK1 and Lgr5 mRNA expression level in CRC patients compared to healthy controls. Data are shown as mean ± SD

showed a higher expression of Lgr5 (p=0.028). In this regard, 66.7 % of poorly differentiated, 50 % of moderately differentiated, and 12.5 % of well-differentiated tumors showed "high expression" of Lgr5 comparatively. Furthermore, Lgr5 expression was higher in neoadjuvant CRT-treated patients (66.6 %) compared to patient with no CRT history (32 %) (p=0.018). The correlation of Lgr5 expression with other clinicopathologic parameters including sex, age, tumor location, and tumor size was not significant. The correlation of DCLK1 and Lgr5 protein expression with patients' clinicopathologic findings has been summarized in Table 2.

Analyzing the results of both CSC markers in paraffin-embedded tissues of CRC patients using Spearman's correlation coefficient test have revealed a significant reciprocal pattern of expression between DCLK1 and Lgr5 (r=0.401, p=0.006).

Table 1 Correlation of clinicopathologic findings with DCLK1 and Lgr5 mRNA expression showed by p value

Parameter	Groups	Number (%)	DCLK1 (p value)	Lgr5 (p value)
Age (years)	20 to 40	9 (15.5)	0.22	0.17
	40 to 60	23 (39.7)		
	≥60	26 (44.8)		
Gender	Female	27 (46.5)	0.09	0.11
	Male	31 (53.5)		
Tumor location	Colon	34 (58.6)	0.086	0.075
	Rectum	24 (41.4)		
TNM stage	I and II	11 (19)	0.015	0.02
	III	28 (48.3)		
	IV	19 (32.7)		
Grade	Well differentiated	8 (13.8)	0.022	0.024
	Moderately differentiated	26 (44.8)		
	Poorly differentiated	24 (41.4)		
Number of metastatic sites	0	39 (67.2)	0.066	0.13
	1	14 (24.2)		
	≥2	5 (8.6)		
Lymphatic invasion	Unknown	7 (12.1)	0.082	0.093
	No	16 (27.5)		
	Yes	35 (60.4)		
Neoadjuvant history	Yes	33 (56.9)	0.01	0.019
	No	25 (43.1)		
Tumor size	<25 mm	32 (55.3)	0.13	0.18
	>25 mm	26 (44.8)		

p Value of <0.05 was considered statistically significant

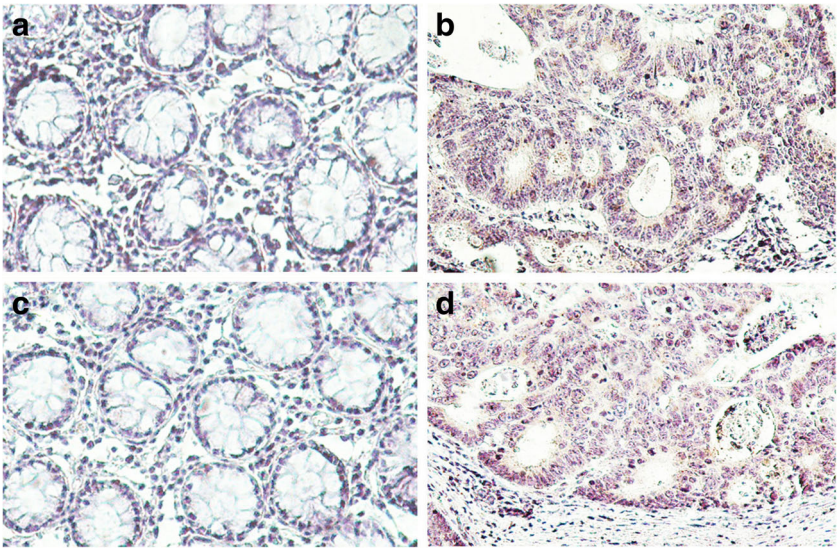

Fig. 2 **a** Weak Lgr5 immunoreactivity in normal colon (H-score=10). **b** Strong Lgr5 immunoreactivity in colon adenocarcinoma (H-score=60). **c** Weak DCLK1 immunoreactivity in normal colon (H-score=5). **d** Strong DCLK1 immunoreactivity in colon adenocarcinoma (H-score=30) (20× magnification)

Protein and mRNA expression level of the markers was significantly correlated

In order to evaluate the correlation trend of protein and mRNA expression level of the markers, Pearson's correlation coefficient test was implemented. The results of correlation analysis showed a significant positive correlation between DCLK1 protein and mRNA expression level (r=0.674, p<0.001). Such positive correlation was also observed between Lgr5 protein and mRNA expression level (r=0.670, p<0.001).

Discussion

In spite of recent evidences supporting the role of DCLK1 and Lgr5 as the most potential colorectal CSC markers [18, 34–36] and their relevance to patient's clinicopathologic findings [37–40], most studies have focused on investigating DCLK1 and Lgr5 expression levels solely in tissue or circulation. Amongst, a higher mRNA expression level of DCLK1 and Lgr5 in the blood circulation of CRC patients compared to tumor-free blood samples was observed in our previous study [22]. Valladares et al. also reported that the expression pattern of circulating Lgr5 was correlated with poorer outcome of CRC patients, metastasis, and high grade of CRC [23].

Despite of the valuable potentiality of CCSC markers in diagnostic and prognostic applications, limited number of researches are devoted to investigate CCSC due to the novelty of this research area and difficulties for CCSC evaluations. Furthermore, most of these studies have applied PCR method to evaluate mRNA expression of the circulating markers, instead of protein assessment which results in more relevant outcomes. Rationally, CSCs constitute a small subpopulation of tumor cells, and from this subset, only a small fraction enter the blood stream which leads to a difficulty in the evaluation of the protein expression of CCSC markers [41]. Nevertheless, higher concentration of CSCs in tumor tissue makes the protein analysis of CSC markers more accessible. Gagliardi et al. evaluated DCLK1 local protein expression in tumor tissues of CRC patients and found a higher expression of DCLK1 in 75 % of primary adenocarcinomas providing insight into its potential prognostic capability [35]. They also demonstrated that 38 % of high-grade tumors were positive for DCLK1, compared to 0 % of low-grade tumors, which confirms our results. Similar to our study, no significant association was found between staining score and tumor location, tumor size, and morphology. In a study carried out by Wu et al., the local protein expression of Lgr5 has been assessed in 192 colorectal carcinoma specimens and revealed a significant higher Lgr5 expression level in carcinoma compared to normal mucosa and a positive correlation of Lgr5 with histological grade and TNM stage which resembled our results [38]. Furthermore, our results showed that local expression of DCLK1 and Lgr5 moves in the same direction at both protein and mRNA level

Table 2 Correlation of clinicopathologic findings with DCLK1 and Lgr5 protein expression showed by p value

Parameter	DCLK1 (p value)	Lgr5 (p value)
Age (years)	0.22	0.27
Gender	0.19	0.17
Tumor location	0.084	0.15
TNM stage	0.014	0.045
Grade	0.023	0.028
Number of metastatic sites	0.12	0.23
Lymphatic invasion	0.09	0.14
Neoadjuvant history	0.016	0.018
Tumor size	0.1	0.083

p Value of <0.05 was considered statistically significant

which further propose that DCLK1/Lgr5 combination marker may hold promise as prognostic markers.

In addition to the simultaneous evaluation of local DCLK1 and Lgr5 proteins, which was performed for the first time in the current study, we also attempted to compare the protein and mRNA expression level of these markers. A considerable amount of previous studies about CSC markers have focused on mRNA evaluation which does not necessarily reflect protein expression level [42, 43]. In support of this, the assessment of protein and mRNA expression level in tumor tissue was perused simultaneously in our study to elucidate the protein and mRNA level of consistency. The observed accordance of protein and mRNA expression level in tumor tissue, although, may reflect such agreement in blood circulation; however, further studies are required to delineate the protein level of circulating CSC markers focusing on designing highly sensitive protein detection assays.

In spite of all revealed positive correlations, some inconsistencies were observed which should be taken into consideration. For example, DCLK1 and Lgr5 mRNA level in some cases were lower than assigned cutoff point, while in some controls, their value crossed the cutoff line. In addition, in some cases, DCLK1 and Lgr5 moved in the opposite directions which also were observed in some controls (Fig. 3). To explain such observations, it should be noted that although DCLK1 and Lgr5 are proposed as the most potential colorectal CSC markers, their expression might be affected by many other factors including genetic and epigenetic background of the patients. In addition to person to person heterogeneity, tumor heterogeneity may also affect the markers' expression [44]. Moreover, high expression of the markers in normal margins may reflect tumor microspread which could be used as an indicator in tumor monitoring.

Furthermore, in some cases and controls, DCLK1 and Lgr5 protein and mRNA did not correlate with each other (Fig. 3). As previously mentioned, mRNA expression does not necessarily convey protein expression. In fact, mRNA may or may not translate into protein for many reasons. As an example, microRNAs (miRNAs) are considered as one of the mRNA degradation factors which inhibit mRNA to protein translation [45]. Nowadays, the result of epigenetic-miRNA interaction is considered as a new layer of complexity in gene regulation which its comprehension will open new avenues to understand human cancerogenesis [46]. Whatever the reason, comprehension of such inconsistencies depends on future complementary studies.

Surprisingly, no study has been performed to compare the local and circulating markers' expression. Based on our hypothesis, comparison of expression level in both tissue and peripheral blood of same patients would lead to a better understanding of CSC markers' expression pattern and provide valuable insight into future CSC diagnostic and prognostic implications. Considering CSC ambiguities such as heterogeneity and plasticity, as missing pieces of a puzzle, exploring the position of each piece and its relevance to other parts will lead to a final configuration of all pieces. In fact, the more CSC complications get unraveled, the sooner CSCs may enter into clinical applications. Therefore, in the present study, we aimed to analyze the DCLK1 and Lgr5 mRNA expression pattern in tumor tissues of CRC patients in order to compare the results with our formerly studied mRNA expression of these markers in blood circulation of same patients [22]. Our real-time PCR finding showed a higher mRNA expression level of DCLK1 and Lgr5 in fresh tumor tissues of CRC patients compared to normal adjacent margins. The overexpression of DCLK1 and Lgr5 was correlated positively with tumor stage and grade. In addition, a positive significant correlation was observed between DCLK1 and Lgr5 mRNA level of CRC tumors. Our current results were in complete accordance with our previous findings, indicating the consistency of circulating DCLK1/Lgr5 expression with their local expression.

In line with our previous data in PB, we found that the mRNA expression level of DCLK1 in CRC patients who received preoperative CRT was higher than patients without history of CRT. Nevertheless, such correlation was not observed between local and circulating Lgr5 expression. Although the level of mRNA expression of LGR5 in neoadjuvant CRT-treated tumor tissues was higher than non-treated patients, such higher expression was not detected in blood circulation of CRT-treated CRC patients [22].

Enrichment of CSCs after CRT treatment has been reported in several studies [47–50]. Hypothetically, following the CRT-imposed shrinkage of tumor bulk and in order to reconstruction of tumor mass, CSCs activate their self-renewal division capability as well as differentiation. This transition from quiescence to activation state leads to the more active CSCs that finally results in faster reconstruction of tumor mass. More interestingly, some evidences suggest that in CRT-treated tumors, dedifferentiation of non-CSCs to CSCs may be rather high [15] leading to a more growing CSC population. Such manifestations provide evidence for CRT-promoting effects on CSC plasticity and heterogeneity which may result in a new CSC makeup [51]. Consequently, the observed difference between local and circulating Lgr5 in CRT-treated patients could be associated to CRT-induced heterogeneity. CRT inducing effect on CSC reprogramming has been reported repeatedly [52–54] giving rise to a new CSC with new combination of markers.

Several additional factors including epithelial-to-mesenchymal transition (EMT) [55–57], different CSC niche [58]. and development of CSC metastatic traits [59] may also lead to different expression of local and circulating CSC markers.

Although CSC heterogeneity has always been regarded as a drawback in CSC investigations, it is of note that from a different prospective, such diversity may be taken as an advantage to help tumor victims. In this regard, finding a difference between local and circulating CSC markers may be

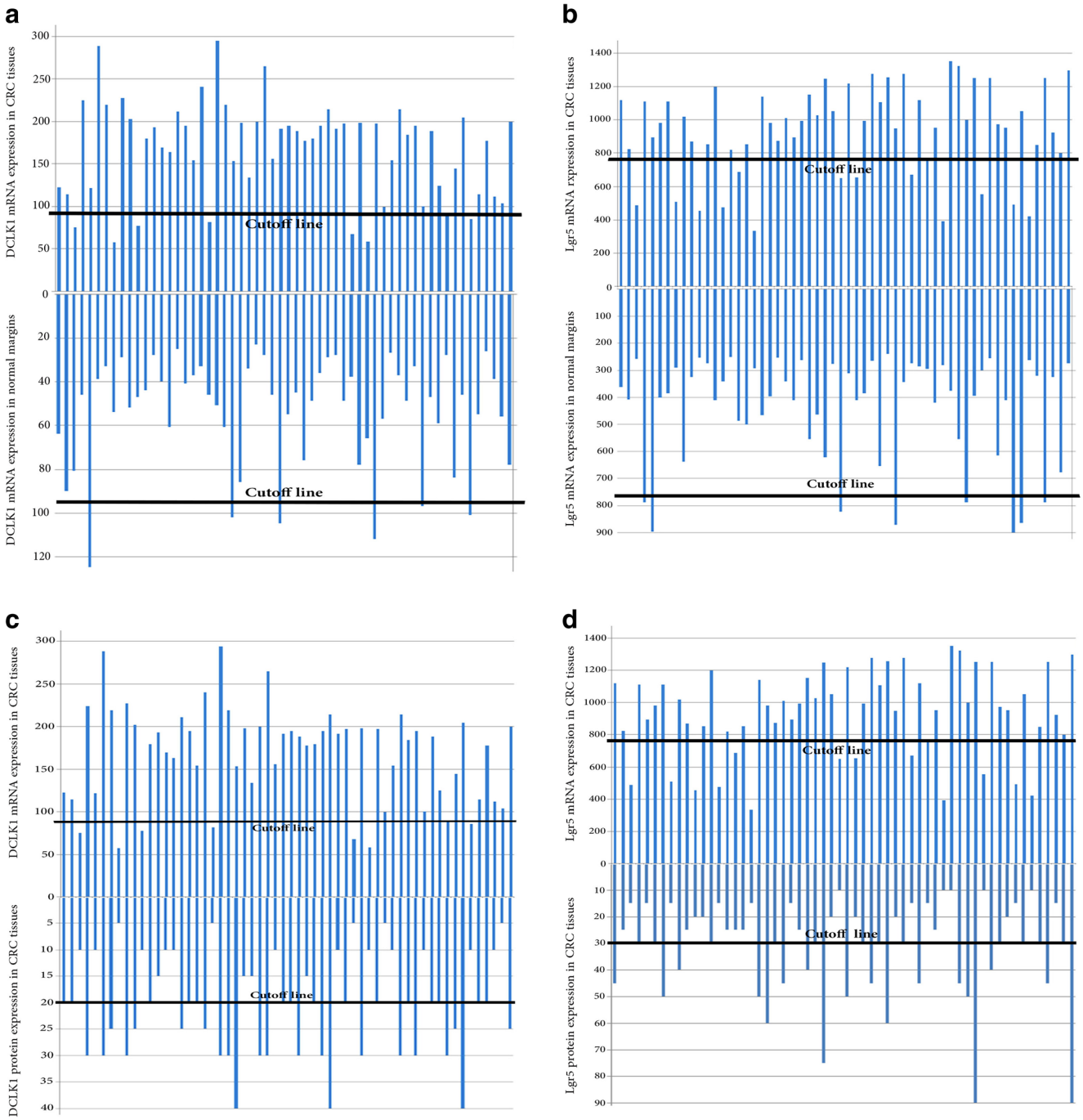

Fig. 3 **a** Comparative graph for DCLK1 mRNA expression level in CRC tissues and normal margins. **b** Comparative graph for Lgr5 mRNA expression level in CRC tissues and normal margins. **c** Comparative graph for DCLK1 mRNA and Pprotein expression level in CRC tissues. **d** Comparative graph for Lgr5 mRNA and prorein expression level in CRC tissues; cutoff line has been drawn to facilitate the comparison of two values

considered as a privilege to target local and circulating CSC differently. Based on this model, in order to prevent tumor metastasis, a circulating CSC marker could be targeted instead of local CSC marker. Moreover, we may search for a local CSC marker in the blood circulation unaware of the fact that not all local CSC markers also work as circulating CSC markers. As a result, exploring potential difference in local and circulating CSC marker expression pattern would be considered as a valuable aspect of heterogeneity that should be more explored in future studies.

Regardless of all potential factors evolving CSC heterogeneity, the observed difference in local and circulating Lgr5 may be simply explained by Nakanishi's description of colorectal CSC. As Nakanishi et al. demonstrated, DCLK1 marks CSCs

and not normal stem cells, while Lgr5 marks both normal and cancer stem cells. Although Lgr5 accompanies DCLK1 on colorectal CSCs, evidences suggest that DCLK1 is a more validated colorectal CSC marker in attribution to tumor characteristics.

Conclusion

Our analysis revealed a significant positive correlation between DCLK1 and Lgr5 at both protein and mRNA expression level. In addition, the mRNA and protein expression of the markers significantly moved in the same direction. Our results also showed a similar local and circulating CSC markers' mRNA expression pattern, with some minor differences such as the higher expression of Lgr5 in local but not circulating CSCs in neoadjuvant CRT-treated CRC patients. Considering CRT as one of the factors promoting CSC heterogeneity, the observed difference in local and circulating Lgr5 expression in CRT-treated patients might be attributed to CRT effect. In addition, several other potential factors may affect the local and circulating CSC markers' combination which their effect remains to be clarified in future studies. Whatever the reason, heterogeneity could be taken as an advantage to help cancer patients; therefore, the old notion about the heterogeneity and its complicating nature needs to be reevaluated.

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards."

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