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Induction of let-7e gene expression attenuates oncogenic phenotype in HCT-116 colorectal cancer cells through targeting of DCLK1 regulation

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ABSTRACT

Aims: MicroRNAs (miRNAs) are small noncoding RNAs that negatively control gene expression at the translational level. There are compelling evidences indicating that the expression of let-7e is downregulated in various cancers, however, the role of let-7e in colorectal cancer (CRC) and its mechanism has been remained unknown. Here, we investigated the potential role of let-7e in regulating CRC cells phenotypes.

Main methods: Let-7e and DCLK1 siRNA were transfected in HCT-116 cells. Colony formation assay, scratch test, Annexin V/PI flow cytometry, and sphere formation assay were performed to examine the cell proliferation, migration, apoptosis, and stemness, respectively. The expression of let-7e, epithelial-mesenchymal transition (EMT)-related genes, Doublecortin like kinase protein 1 (DCLK1), and cancer stem cells (CSCs) were assessed using RT-qPCR while the protein level of DCLK1 was determined by western blotting.

Key findings: Overexpression of let-7e effectively inhibited cell proliferation, suppressed migration, reduced sphere formation, and precluded EMT process as well as stemness factors. Furthermore, let-7e suppressed DCLK1 expression. Additionally, we found that the expression of let-7e was negatively correlated with DCLK1 expression in CRC cells.

Significance: Let-7e plays an important role as tumor suppressor miRNA in CRC probably through inhibition of DCLK1 expression.

1. Introduction

Colorectal cancer (CRC) is the third most frequent cancer diagnosed in men (after lung and prostate cancer) and the second in women (after breast cancer) around the world, with a prevalence of 10% and 9.2%, for males and females, respectively [1]. Despite the advances made in the diagnosis and treatment of CRC, this cancer is still one of the causes of death, mainly due to resistance to chemotherapy and radiation therapy [2].

MicroRNAs (miRNAs) are tiny non-coding RNAs (18–22 nucleotides) which suppress the translation of mRNA into proteins via interacting with their 3′ untranslated regions [3]. Each miRNA is able to regulate the expression of several genes and influences cancer stem cells (CSCs) by playing multiple roles in biological processes including cell survival, proliferation, metastasis, and chemoresistance [4]. miRNAs fall into two categories, oncomiRs or tumor suppressor miRNAs based on their mRNA target. In recent years, it has been shown that aberrant expression of various miRNAs is related to cancer progression, migration, growth, proliferation, etc. [5–8].

Lethal-7 (*let-7*) was discovered as the first human miRNA in the *Caenorhabditis elegans* [9]. Ten members of the human let-7 family have been identified, including *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7g*, *let-7i*, *miR-98* and *miR-202* [10]. Let-7 family is highly conserved in many species such as humans with a critical role in normal cellular development, growth, and differentiation [11].

It has been shown that let-7e is one of the downregulated miRNAs in various cancer types such as gastric adenocarcinoma, prostate cancer, ovarian cancer, breast cancer, and CRC compared with normal cells and tissues [12]. Although the functional role of let7-e in CRC has not been fully understood, but recent studies have shown that downregulation of let-7e is associated with tumor progression, metastasis, chemotherapy resistance and radioresistances [13–17].

Doublecortin like kinase protein 1 (DCLK1) is a potent marker for gastrointestinal stem cells and involved in cancer cell survival, proliferation, invasion, metastasis and EMT [18,19]. We found that DCLK1 is a target gene of let-7e and there was a negative correlation between let-7e and DCLK1 expression. This new mechanism might be considered as a new therapeutic target for CRC treatment.

In the present study, we showed that the let-7e overexpression in HCT-116 CRC cell line by targeting DCLK1 is associated with decreased

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Table 1
Primer sequences for qRT-PCR.

Gene	Sense strand	Antisense strand
DCLK1	TTGCTCCAGATCGTTAGAAGG	CAGGAAGGTCTCATTGAACAC
CD133	GAGTCGGAAACTGGCAGATAG	AACGCCTTGTCCTTGGTAG
BMI1	CATCCACAGTTTCCTCACATTTC	GAAGTTGCTGATGACCCATTTAC
CD44	AATGGTCGCTACAGCATCTC	GCCCTTCTATGAACCCATACC
ALDH1	GCCAGGTAGAAGAAGGAGATAAG	CTCGGAAGCATCCATAGTACG
E-cadherin	AGAACGCATTGCCACATACA	GAGGATGGTGTAAGCGATGG
Vimentin	CATTGAGATTGCCACCTAC	CGTTGATAACCTGTCCATC
N-cadherin	ATTCGGGTAATCCTCCCAAATC	CCCACAATCCTGTCCACATC

cell proliferation, migration, CSC- like properties, and epithelial-mesenchymal transition (EMT).

2. Materials and methods

2.1. Cell culture and transfection

Human CRC cell line HCT-116, were purchased from the National cell bank of Pasteur Institute (Tehran, Iran). HCT-116 cells were cultured in Dulbecco's modified Eagle's medium (*DMEM*), containing 10% fetal bovine serum (*FBS*), and 1% Penicillin-Streptomycin and were maintained at 37° in a humidified chamber with 5% CO_2. In order to transfect cells with let-7e miRNA mimic and DCLK1 siRNA, cells were seeded in 6-well plate (5×10^5 cells) for 24 h to reach 70% confluency. Next, cells were transfected with let-7e miRNA mimic and DCLK1 siRNA using HiPerFect Transfection Reagent from Qiagen (USA), according to the manufacturer's instructions. Cells were incubated for 24 h and then harvested for further experiments.

2.2. Colony formation assay

HCT-116 cells were plated in 6-well plates and were transfected with let-7e mimic. After 7 days, colonies were stained with crystal violet and the cell colonies were counted.

2.3. RNA isolation and RT-qPCR

Total cellular RNA was extracted by RNX-Plus kit (Cinnagen, Tehran, Iran) according to the manufacturer's directions, and RNA purity and concentration were assessed using NanoDrop (Thermo Fisher Scientific, Waltham, MA). First strand cDNA for miRNA and mRNA was synthesized using the miRCURY LNA™ Universal RT cDNA Synthesis Kit (Exiqon, Denmark) and First Strand cDNA Synthesis Kit (Fermentas, USA), respectively. Then, the expression measurements of miRNA and genes was done by ExiLENT SYBR® Green master mix (Exiqon, Denmark) and SYBR Premix Ex. Taq II master mix (Takara), respectively. Analysis of data was done by normalizing with GAPDH and U6 internal controls, and relative expression of genes was determined using the $2^{-\Delta\Delta CT}$ method. List of primer sequences used for RT-qPCR is listed in Table 1.

2.4. Protein extraction and Western blot analysis

After harvesting HCT-116 cells, total protein was extracted using RIPA buffer (Santa Cruz, USA) plus protease inhibitors. In order to determine total protein concentration, the Bradford protein assay was used. After that, by using 10% SDS-PAGE the equivalent volumes of proteins were separated and transferred onto a nitrocellulose membrane. Then, by blocking the membrane with 5% dry milk, the membrane was incubated with anti-DCLK1 (1:1000, Abcam, ab37994) and GAPDH (as the internal control, 1:10,000, Abcam, ab37168) antibodies at 4 °C overnight. The secondary antibody conjugated to horseradish peroxidase was used to visualized the bands with ECL detection kit. Finally, bands were quantified by ImageJ software.

2.5. Sphere formation assays

For determine the HCT-116 sphere forming activity, cells were plated in a 6-well low attachment plate containing 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (b-FGF). Cells were incubated at 37 °C and 5% CO_2 for 7 days and the number of tumor spheroids were counted in all groups under an inverted microscope.

2.6. Migration assay

The ability of migration of HCT-116 cells was examined by scratch assay. Briefly, 2×10^5 cells were seeded in the 24 well plates and incubated at 37 °C for 8 h. Then, the culture area was scratched with a crystal pipette tip to make a linear gap in the confluent cell monolayer. Finally, cells were allowed to fill the gap and images of the culture area were captured at regular time intervals of 24 h.

2.7. Flow cytometry analysis of apoptosis

1×10^6 HCT-116 cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) at room temperature in dark conditions. Finally, flow-cytometry was performed using a FACS Canto II instrument and data were analyzed using PartecFloMax software (USA).

2.8. Statistical analysis

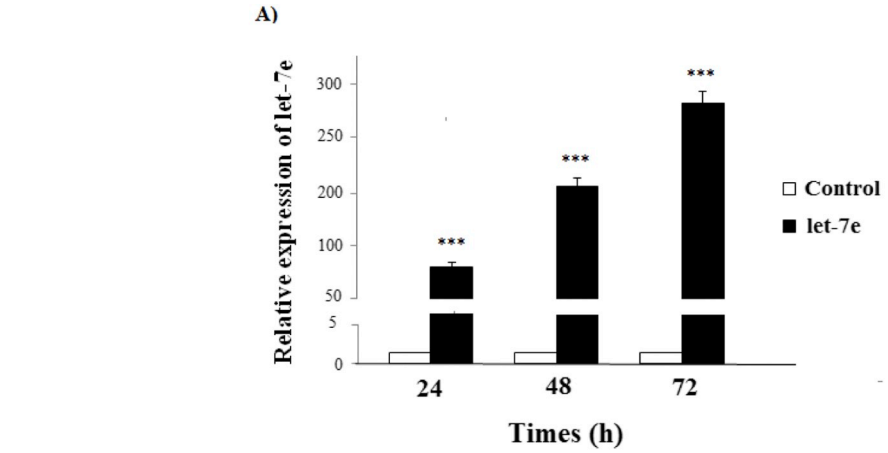
SPSS 16.0 software was used for data analysis. One-way ANOVA followed by Tukey test was performed to determine differences between groups. Data were presented as the mean ± standard error (SEM) and the P value of < 0.05 was considered statistically significant.

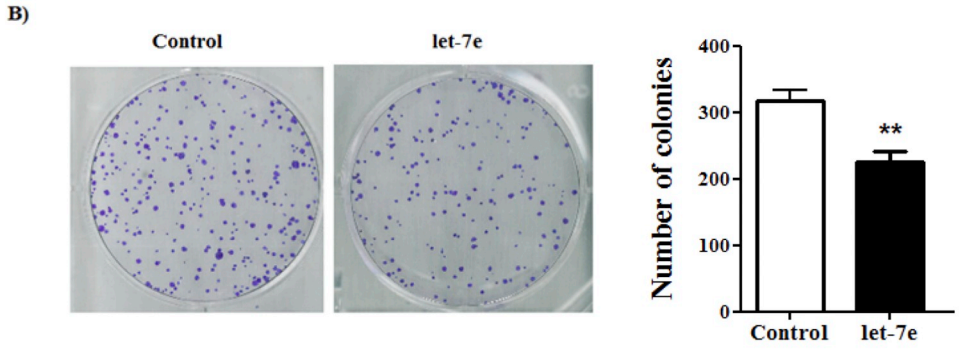
3. Results

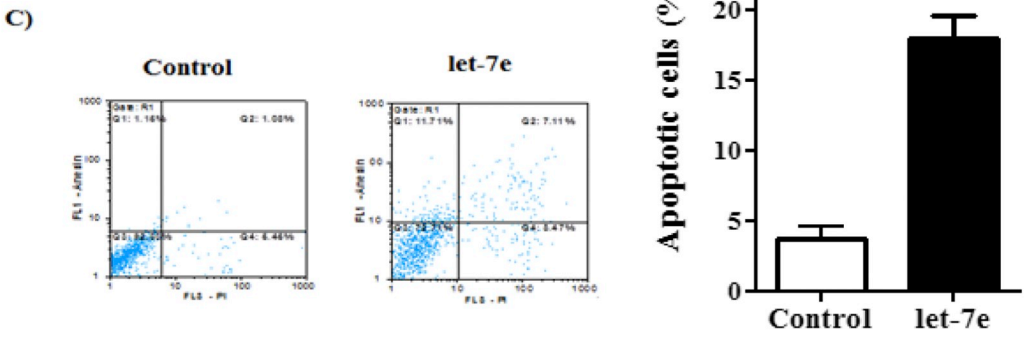
Let-7e inhibits the proliferation and migration of CRC cells and induces apoptosis.

Previous studies showed that let-7e is downregulated in CRC and recent reports suggested that downregulation of let-7e is associated with tumor progression and metastasis. However, the functional role of let-7e is unclear. To understand how the expression of let-7e affects the proliferation and migration of CRC cells, HCT-116 cells were transfected with let-7e mimic and transfection efficiency was evaluated using RT-qPCR. Let-7e expression level was significantly increased after 24, 48 and 72 h following let-7e mimic transfection compared with that in untransfected cells (Fig. 1a).

As the capacity of cells to proliferate and produce colonies is a key characteristic of cancer cells, we examined the colony forming abilities of let-7e transfected HCT-116 cells. Restoration of let-7e significantly reduced the number and size of colonies compared to control group (Fig. 1b). Furthermore, the effect of let-7e on apoptosis was evaluated using the Annexin V/PI assay. The apoptosis assay showed that let-7e-







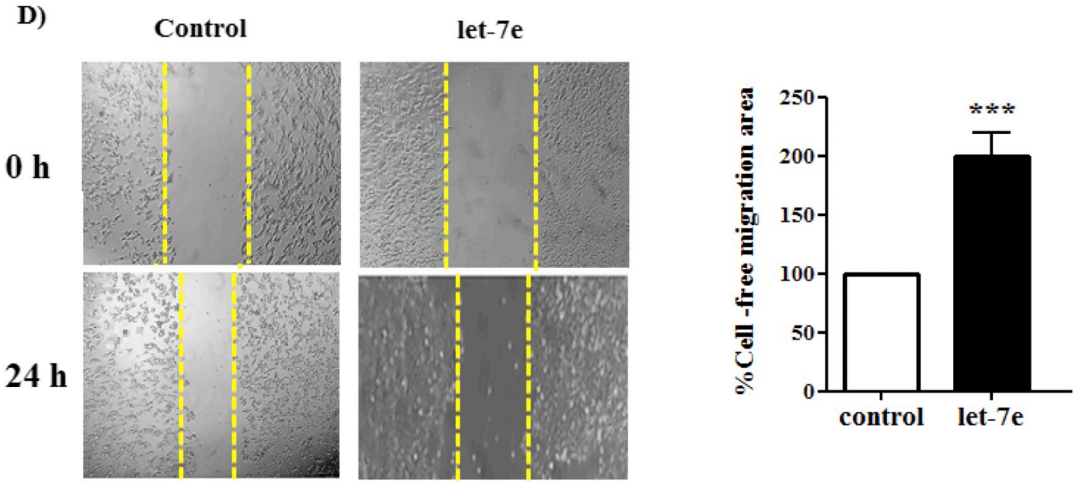


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Fig. 1. Upregulation of let-7e inhibits colon cancer cell proliferation and migration. A) After transfection with let-7e, the levels of let-7e were detected by RT-qPCR. U6 was used as the internal control. B) The effects of let-7e mimics on the proliferation of HCT-116 cells were detected by colony formation assay. C) The effect of re-expression of let-7e on apoptosis was analyzed by flow cytometry. D) The migration capability of HCT-116 cells was evaluated by a scratch assay after transfection with let-7e mimics. Results are expressed as the mean ± SEM (n = 2). $^{**}P < 0.01$ and $^{***}P < 0.001$.

overexpressing HCT-116 cells exhibited a considerable increase in the percent of apoptotic cells compared with that of untransfected cells (Fig. 1c).

In order to investigate whether let-7e can affect the migration ability of HCT-116 cells, let-7e transfected cells were grown in 24-well plates, then wound was created and cells allowed to migrate for 24 h. Quantification of cell-free area showed a significant inhibition of cell migration in HCT-116 cells transfected with let-7e mimic (Fig. 1d).

3.1. Let-7e attenuates EMT and CSC characteristics

Many studies clearly show that EMT process and CSCs play important roles in cancer initiation, tumor recurrence, radioresistance, and chemoresistance. Therefore, we investigated the effects of let-7e on the regulation of EMT markers and CSCs activities. HCT-116 cells were transfected with let-7e mimic. RT-qPCR was performed to determine gene expression levels of EMT markers (E-cadherin, vimentin, and N-cadherin). As presented in Fig. 2a, overexpression of let-7e in HCT-116 resulted in downregulation of the mRNA expression of mesenchymal markers vimentin and N-cadherin compared with control groups, while the expression level of epithelial marker E-cadherin was significantly increased compared with the untransfected group.

To determine the effects of let-7e on CSCs phenotype in CRC, we transfected the HCT-116 cells with let-7e mimic and the expression of CSCs markers including CD44, ALDH1, CD133, and BMI1 were analyzed by RT-qPCR. We found that CSCs mRNA was significantly inhibited by let-7e in transfected HCT-116 cells, as compared to control groups (Fig. 2b).

The important feature of CSCs is their ability to form sphere when they grow in serum-free medium in non-adherent plates. To further confirm that CSCs markers can be inhibited by let-7e, we performed sphere formation assay to examine the capacity of CSCs self-renewal in HCT-116 cells. The results showed that the size and sphere number in let-7e overexpressing cells was much lower after 7 days compared with control cells (Fig. 2c).

3.2. The reverse relationship between let-7e and DCLK1 expression in CRC cells

DCLK1 is a putative CSCs marker especially for pancreas and the small intestine [20,21]. To elucidate the possible mechanism by which let-7e regulated the HCT-116 tumorigenesis, we examined the effect of let-7e overexpression on DCLK1 gene and protein level. As presented in Fig. 3a, the relative expression of DCLK1 was significantly decreased in HCT-116 cells transfected with let-7e mimic compared with that in the control group.

In our previous research, we studied the properties of HCT-116 cells after DCLK knocking down with siRNA. DCLK1 can regulate critical miRNAs that play important roles in oncogenic pathways such as survival, EMT, and CSCs. For example, recent studies indicated that downregulating DCLK1, increased the expression of *miR-200a*, *b*, *c*, miR-144, *miR-143/145*, and *let-7a*. So to clarify whether the DCLK1 could affect the let-7e expression, the expression of DCLK1 in HCT-116 cells was silenced using siRNA. The transfection efficiency was approved using RT-qPCR and Western blot methods. As described in Fig. 3b, DCLK1 siRNA downregulated the expression of DCLK1 gene and protein.

Similarly, here we observed a considerable induction of let-7e expression following the knockdown of DCLK1 in HCT-116 cells (Fig. 3c). According to these results, we speculate that there is a negative feedback loop mechanism between let-7e and DCLK1.

4. Discussion

miRNA's dysregulation is involved in various diseases such as cancers. miRNAs have essential roles in cancer cell development, differentiation, invasion, and metastasis. Therefore, they can be considered as potential diagnostic markers [22]. miRNAs are able to trigger translational repression or mRNA degradation via binding to complementary sequences in the 3′-UTR of their target genes. It seems that miRNAs can regulate about 30% of the transcriptome [23].

Generally, the role of the let-7 family of miRNAs in cancer development has been demonstrated in many cancer types. For instance, certain studies have shown that let-7a miRNA inhibits tumor invasion and metastasis in thyroid carcinoma [24], breast cancer [25], and nasopharyngeal carcinoma [26]. Furthermore, in other studies let-7b, let-7f, let-7g have also exhibited important roles in tumor growth, proliferation, migration, and invasion of gastric cancer [27], glioma [28], and hepatocellular carcinoma [29], respectively.

In relation to let-7e, the studies in cancer are relatively rare, especially in CRC. Previous studies have shown that let-7e down regulated in CRC tissues compared with adjacent non-cancerous tissues and overexpression of let-7e reduced cell proliferation and invasion via IGF-1R and Akt inhibition [30,31]. Man Xiao et al. reported that decreased let-7e expression is associated with chemoresistance in ovarian cancer by the activation of Rad51 and BRAC1 expression [16]. Another evidence shows a link between let-7e and radiosensitivity of CRC cells through targeting of IGF-1R [32].

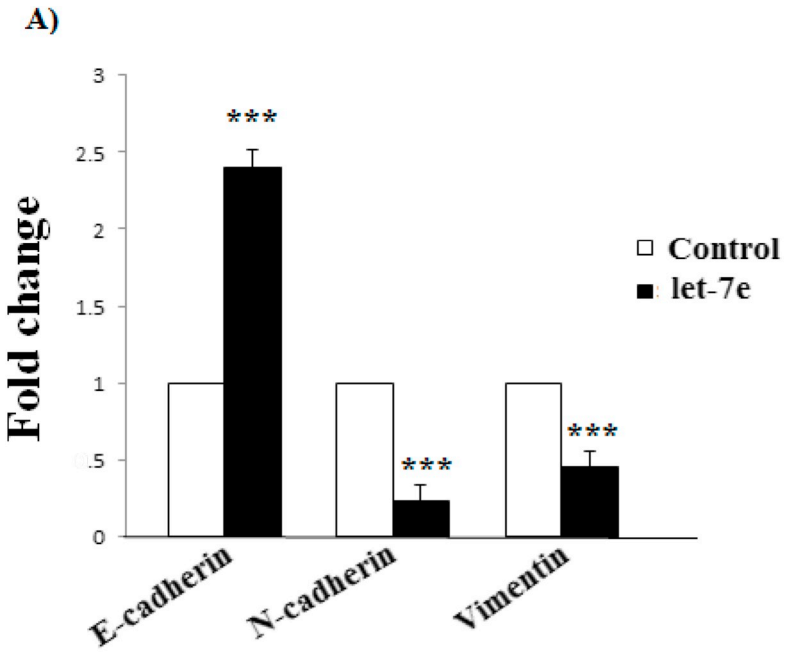
However, the molecular mechanism by which let-7e exerts its tumor suppressor role in CRC remains unknown. Therefore, in the present study we aimed to clarify the function and mechanism of let-7e in CRC.

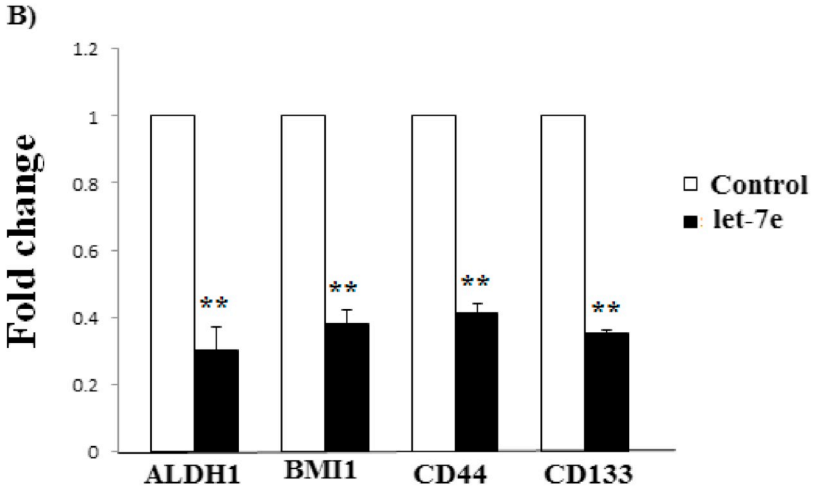
The current study revealed that the ectopic expression of let-7e inhibited HCT-116 cells proliferation and migration, and induced apoptosis. In addition, the gene expression of EMT-related markers including vimentin, N-cadherin and E-cadherin and CSCs were significantly affected by let-7e. These data suggested that let-7e functions as a tumor suppressor miRNA in CRC. As reported earlier, let-7 regulates stemness and EMT through blocking self-renewal and accelerating differentiation of CSCs [31,33].

To investigate the underlying molecular mechanism of let-7e in CRC tumorigenesis, we examined the impact of let-7e on a specific colorectal CSC marker, DCLK1. RT-qPCR analysis showed that DCLK1 gene expression is dramatically decreased in let-7e overexpressing HCT-116 cells.

We previously reported that DCLK1 was up-regulated in CRC tissues compared with normal colon tissues, also, DCLK1 silencing with siRNA in HCT-116 and SW-48 cell lines reduced their invasion and migration capabilities and triggers apoptosis by inhibiting miR-200c [34].

DCLK1 is a serine/threonine kinase and a member of microtubule-associated proteins which proved itself as a validated CSCs marker in many cancer types including CRC [35]. Different studies have demonstrated that DCLK1 dysregulation plays important roles in motility, growth, invasion, and EMT of cancer cells [36,37]. Overexpression of DCLK1 has been identified to induce development, progression, and migration in a variety of human tumors including salivary gland malignancies, prostate cancer, pancreatic cancer, bladder cancer, ovarian cancer, and CRC [21,38–42]. In addition, DCLK1 has critical roles in facilitating tumorigenesis in *intestine* via increasing EMT and pluripotency factors. Therefore, targeting DCLK1 with siRNA decreases the dysregulation in correlated miRNAs, EMT markers, and pluripotency





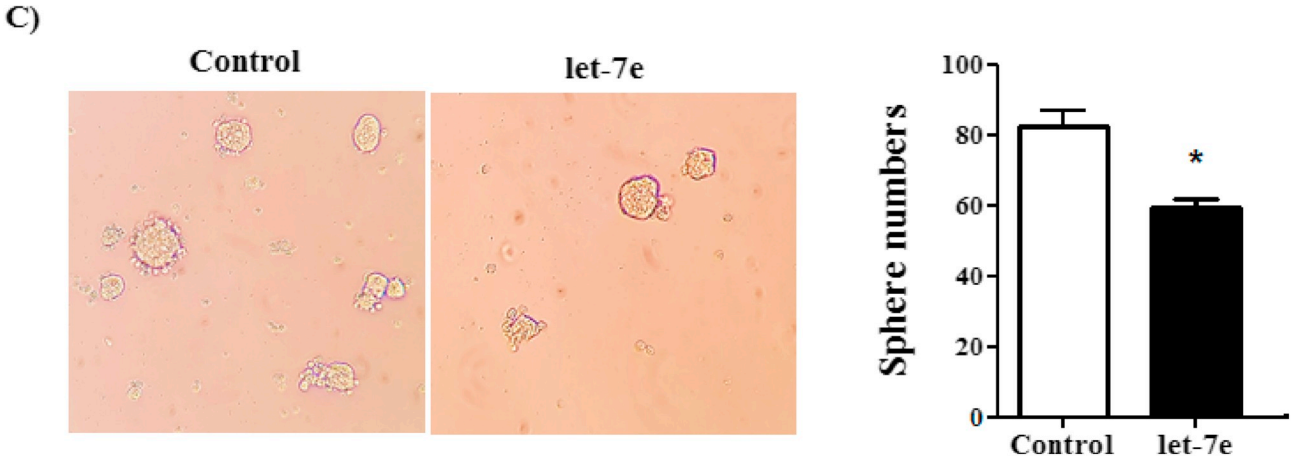


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Fig. 2. Transient expression of *let-7e* inhibits the expression of EMT markers and CSCs in HCT-116 cells. A) RT-qPCR analysis of EMT related markers in cells transfected with let-7e or control. B) The mRNA expression of CSCs factors ALDH1, BMI1, CD44 and CD133 in the cells was detected by RT-qPCR. GAPDH was used as an internal control. C) representative image of sphere formation after 7 days. Results are expressed as the mean ± SEM (n = 2). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

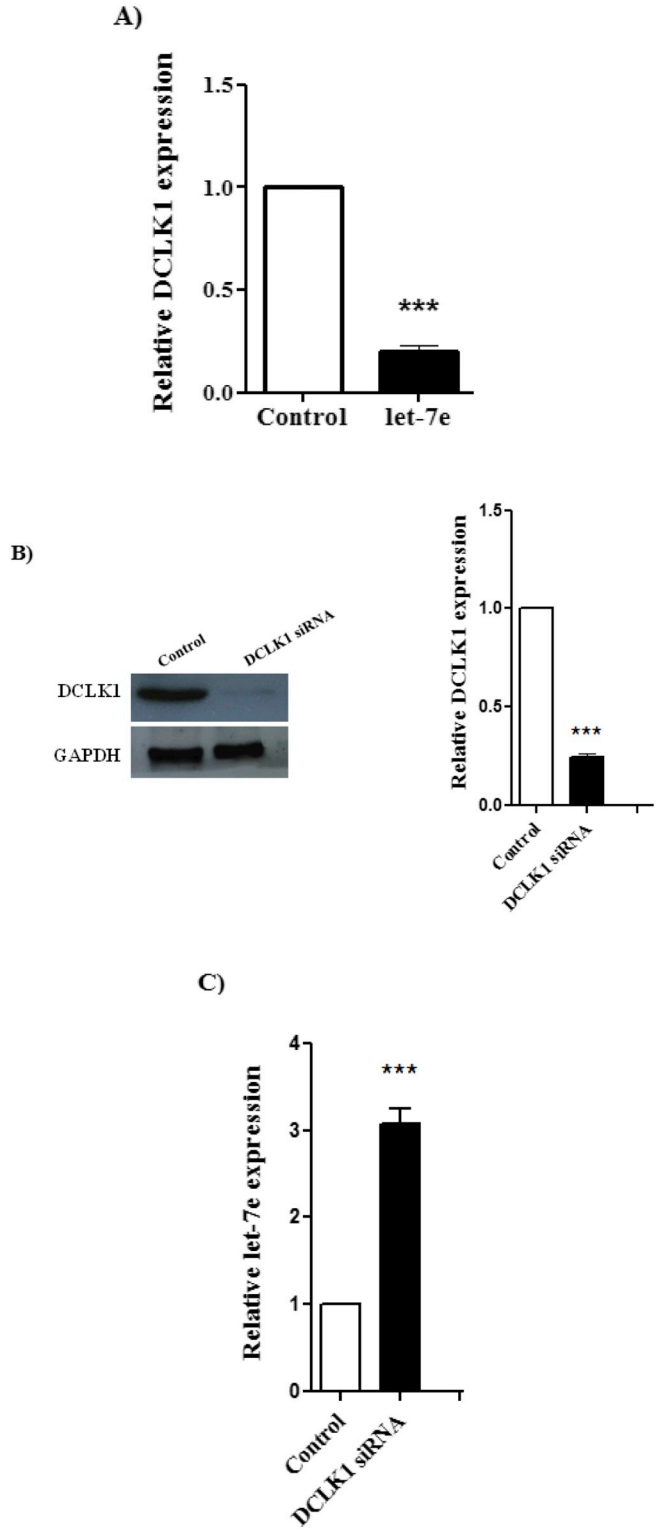

Fig. 3. There is a negative correlation between let-7e and DCLK1 expression. A) The relative expression of DCLK1 in HCT-116 cells transfected with let-7e mimics and control. B) HCT-116 cells were transfected with DCLK1 siRNA and tested for the gene and protein expression of DCLK1. C) RT-qPCR analysis of let-7e expression in cells transfected with DCLK1 siRNA and untransfected cells. Results are expressed as the mean ± SEM (n = 2). $^{***}P < 0.001$.

associated with susceptibility to cancer [43]. These findings suggest DCLK1 as a good candidate of therapeutic strategies for intestinal and/or other solid tumors in patients even with advanced cancer [44].

Recent studies have displayed that DCLK1 is the direct target for several miRNAs such as miR-137, miR-195 and miR-424-5p [45–47]. Here we found that DCLK1 is a possible mediator of let-7e effects and let-7e downregulation in CRC might lead to enrichment of CSCs by increasing of DCLK1 level.

Finally, we investigated the relationship between let-7e and DCLK1 expression. The effect of DCLK1 on let-7e expression was assessed using RT-qPCR. We found in DCLK1-depleted cells the expression of let-7e was increased [45]. These results are in agreement with previous studies indicating that DCLK1 performs its regulatory role in cancer through miRNAs dependent mechanisms. For example, let-7a, miR-144, miR-200a and miR-200c were significantly affected by DCLK1 [34,48,49].

These results confirmed our hypothesis that let-7e negatively regulates the CRC cells proliferation, migration, EMT, and CSCs characteristics through negative feedback regulation of DCLK1. However, in the present study, we did not find let-7e binding site of DCLK1 mRNA using bioinformatics tools and the mechanism of the inhibitory effect of let-7e on DCLK1 expression requires more study to do.

In conclusion, this study demonstrated that let-7e acts as tumor suppressor miRNA in CRC. In addition, let-7e indirectly inhibits the gene expression of DCLK1. Our results suggest that let-7e could be a novel therapeutic target for CRC treatment.

Acknowledgments

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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