Oncogene Lin28B increases chemosensitivity of colon cancer cells in a let-7-independent manner

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Abstract. Lin-28 homolog B (Lin28B) is a RNA binding protein conserved between *Caenorhabditis elegans* and humans, and it has important roles in regulating development. The overexpression of Lin28B has been observed in various human malignant tumors and the upregulation of Lin28B predicts tumor progression and/or poor prognosis. The majority of studies suggested that Lin28B is an oncogene that promotes the proliferation and metastasis of cancer cells. However, few studies have focused on the function of Lin28B in chemotherapy. In the present study, the role of Lin28B in the chemosensitivity of colon cancer cells to 5-fluorouracil (5-FU) was detected by establishing a Lin28B over-expressing HCT116 (EGFP-Lin28B-HCT116) cell line. In accordance with the immunohistochemistry results, Lin28B-GFP expression was predominantly distributed in the cytoplasm, and the overexpression of Lin28B was confirmed using quantitative polymerase chain reaction and western blot analysis. The control EGFP-HCT116 and Lin28B over-expressing EGFP-Lin28B-HCT116 cells were then exposed to various concentrations of 5-FU for 48 h. A luminescence-based cell viability assay was used to detect the effect of Lin28B on the chemotherapeutic sensitivity of colon cancer cells. It was demonstrated that overexpression of Lin28B improved the chemotherapeutic sensitivity of colon cancer cells to 5-FU. Additional investigation revealed that Lin28B enhanced the chemosensitivity of colon cancer cells by promoting cell apoptosis induced by 5-FU; however, this effect was independent of Lin28B inhibiting the biogenesis of let-7, the well-known target of Lin28B. The mechanism of this effect of Lin28B on the chemosensitivity of cells requires additional investigation. The present study suggested that Lin28B may act as a biomarker for predicting chemotherapy sensitivity in patients with colon cancer.

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Introduction

Colorectal cancer is one of the most common malignant tumors worldwide. However, the treatment of colon cancer remains unsatisfactory due to chemoresistance (1).

Lin-28 homolog B (Lin28B) is a conserved RNA-binding protein. It was originally identified in hepatocellular carcinoma, where the protein encoded by this gene is highly increased (2). This protein has been identified to majorly distribute in the cellular nucleus (3). Previously, in addition to hepatocellular carcinoma, numerous studies have indicated that Lin28B is frequently upregulated in various cancers, including colon cancer, and the elevated expression of the protein is associated with advanced tumor stage and poor prognosis (4-6). The mechanisms involved in the promotion of the development of human malignant tumors by upregulated Lin28B include promoting cellular proliferation, angiogenesis and metastasis, facilitating tumor-associated inflammation, reprogramming metabolism, acquiring immortality and evading immune destruction. This may occur by either repressing the generation of let-7, a well-studied miRNA with tumor suppressor function, or stabilizing the oncogenic transcripts, such as cell cycle proteins, cyclin-dependent kinases, growth factors and ribosomal proteins (7).

Although it has been reported that Lin28B promotes colon cancer growth, migration and recurrence (8,9), the function of Lin28B in colon cancer chemotherapy remains unknown. In the present study, the expression of Lin28B in colon cancer tissues and the effect of Lin28B on the chemosensitivity of colon cancer cells to 5-fluorouracil (5-FU) were detected. Additionally, the potential mechanism of Lin28B affecting chemotherapeutic sensitivity of colon cancer cells to 5-FU was also explored.

Materials and methods

Cell culture. The colon cancer HCT116 cell line and human embryonic nephral epithelia 293TN cell line, which was used for lentiviral vector packaging, were maintained in Dulbecco's modified Eagle medium (HyClone; GE Healthcare Life

Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2.

Human tissues and immunohistochemistry. A total of 103 formalin-fixed paraffin-embedded tissues, consisting of 92 colon cancer tissues and 11 adjacent mucosa tissues obtained from colon cancer patients that underwent resection of colon carcinoma at the Affiliated Tumor Hospital of Harbin Medical University between May 2011 and July 2012. The diagnoses for all samples were confirmed by two or more pathologists. The present study was approved by the Ethical Committee of Harbin Medical University (Harbin, China). All patients provided informed consent prior to surgery.

Immunohistochemistry was used to detect the expression of Lin28B in colon cancer tissues or adjacent colon tissues. Briefly, 5-μm-thick sections were prepared from paraffin-embedded tissues. Sections were rehydrated using a gradient alcohol series, as follows: 100% ethanol for 10 min twice; 95% ethanol for 5 min; 80% ethanol for 5 min; and 70% ethanol for 5 min at room temperature. Next, the sections were placed in citrate buffer (pH 6.0) and heated in a microwave oven for antigen retrieval (95˚C for 3 min). Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide for 15 min. Following blocking with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature, sections were incubated with primary antibodies against Lin28B (1:1,000; cat. no. ab115698; Abcam, Cambridge, MA, USA), overnight at 4˚C. Subsequently, the sections were incubated with the secondary streptavidin-biotin peroxidase-conjugated antibody (1:1,000; cat. no. ab6721; Abcam) for 1 h at room temperature, and 3,3'-diaminobenzidine (Origene Technologies, Inc., Beijing, China) was used to stain the sections. The evaluation of Lin28B expression was performed by a pathologist under light microscopic observation (magnification, x400), as previously described (10).

Pseudo lentivirus packaging and transduction into cells. Lin28B over-expressed lentiviral vectors were constructed and the pseudo lentivirus was packaged as described previously (11). To generate Lin28B overexpression of lentiviral vectors, the open reading frame of Lin28B was amplified by 2X EasyTaq PCR SuperMix for PAGE (Beijing Transgen Biotech Co., Ltd., Beijing, China) from HCT116 cells. The polymerase chain reaction (PCR) assays using DNA from HCT116 cells were performed at 95˚C for 5 min and then 40 cycles of denaturation at 95˚C for 10 sec, amplification at 60˚C for 30 sec and extension at 72˚C for 1 min. The DNA polymerase was purchased from Beijing Transgen Biotech Co. Ltd. (2X EasyTaq PCR SuperMix; cat. no. AS111-11; Beijing, China). Primer sequences are summarized in Table I. Subsequent to sequencing and digestion with *Bam*H1 and *Mlu*1, the vector was inserted into a pWPXL lentiviral construction plasmid (cat. no. 12257; Addgene, Inc., Cambridge, MA, USA). The recombinant plasmid and packaging plasmids psPAX2 (cat. no. 12260; Addgene) and PMD.2G (cat. no. 12259; Addgene) were co-transfected into 293TN cells. The transfection was mediated using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h subsequent to transfection, the supernatant was collected and the lentivirus was condensed using polyethylene glycol reagent (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. To generate cells with stable Lin28B overexpression, HCT116 cells were incubated with 10 MOI of control lentiviral particles or Lin28B overexpression lentiviral particles for at least 12 h at 37˚C in a humidified atmosphere containing 5% CO_2, subsequent to culturing for at least 72 h. GFP-positive cells were then identified by assessing the relative fluorescence intensities using the FACSAria II flow cytometer and analyzed with FACSDiva software 6.1.3 (BD Biosciences, Franklin Lakes, CA, USA).

RNA isolation and quantitative PCR. Total RNA was extracted from HCT116 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized by using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (12), with random primers or specific miRNA reverse transcription primers. The relative RNA expression was detected using the SYBR Green quantitative PCR reagent (Beijing Transgen Biotech, Inc.). Gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (13). The PCR reactions consisted of the following conditions: 95˚C for 5 min, followed by 40 cycles of amplification at 95˚C for 10 sec and 60˚C for 30 sec. The primer sequences used for reverse transcription and quantitative PCR are stated in Table I.

Transfection. A total of 50 nM of microRNA negative control (NC; sequence, ucaccuagaagcuaguuaacag) and let-7 mimic (sequence, ugagguaguagguuguauaguu) were synthesized by GE Dharmacon (GE Healthcare Life Sciences). Transfection was performed using GE Dharmacon DharmaFECT kb DNA transfection reagent (GE Healthcare Life Sciences), according to the manufacturer's protocol.

Cell viability assay. In total, $5x10^3$ HCT116 cells were seeded onto a 96-well plate and treated with 80, 160, 320 and 640 μM 5-FU (Jinyao Amino Acid Co., Ltd, Tianjin, China) for 48 h. Cellular viability was measured using CellTiter-Glo reagent (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. Briefly, 100 μl CellTiter-Glo reagent was added to each well, and the plate was equilibrated at room temperature for 30 min. The contents were then mixed on an orbital shaker. Finally, the value of luminescence was measured at 460 nm, and the value was normalized to blank control.

Apoptosis analysis. Cell apoptosis was detected using the Phycoerythrin (PE)-Annexin V Apoptosis Detection kit (BD Biosciences), according to the manufacturer's instructions. Briefly, $1x10^6$ cells were collected, and then washed with PBS and re-suspended in1mlbinding buffer. Subsequently, 100 μl solution ($1x10^5$ cells) was transferred to a 5-ml culture tube, and 5 μl PE-annexin V and 5 μl 7-ADD were then added to the tube. The cells were vortexed gently and incubated for 10 min in the dark at room temperature. Finally, 400 μl binding buffer was added to each tube before analyzing the tubes by flow cytometry.

Western blot analysis. The total proteins were extracted from cells using radioimmunoprecipitation assay lysis buffer

Table I. Primer sequences used in the present study.

Primer name	Primer sequence (5'-3')
Let-7a RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaactat
Let-7b RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaaccac
Let-7c RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaaccat
U6 RT	AAAATATGGAACGCTTCACGAATTTG
Let-7a quantitative PCR	
Forward	TGAGGTAGTAGGTTG
Reverse	GTCGTATCCAGTGCAGGGTCCGAGGT
Let-7b quantitative PCR	
Forward	TGAGGTAGTAGGTTGTGT
Reverse	GTCGTATCCAGTGCAGGGTCCGAGGT
U6	
Forward	CTCGCTTCGGCAGCACATATACT
Reverse	ACGCTTCACGAATTTGCGTGTC
Let-7c quantitative PCR	
Forward	TGAGGTAGTAGGTTGTATt
Reverse	GTCGTATCCAGTGCAGGGTCCGAGGT
GAPDH	
Forward	ATGGGGAAGGTGAAGGTCG
Reverse	GGGGTCATTGATGGCAACAATA
Lin28B	
Forward	GTCAATACGGGTAACAGGAC
Reverse	TTCTTTGGCTGAGGAGGTAG

RT, reverse transcription; PCR, polymerase chain reaction; Lin28B, Lin-28 homolog B.

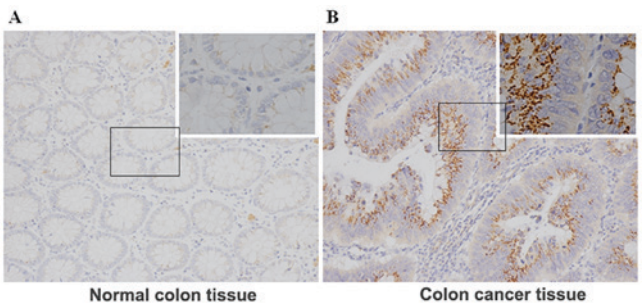

Figure 1. Lin28B was upregulated in colon cancer tissues, as detected by immunohistochemical staining (magnification, x100). The magnification in the top-right corner of the images is x400. (A) Lin28B was not expressed in adjacent colon tissue. (B) Lin28B was expressed in colon cancer tissue. Lin28B, Lin-28 homolog B.

(Beyotime Institute of Biotechnology, Shanghai, China), and subsequently quantified using the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). In total, 20 μg protein/well was loaded and separated by SDS-PAGE (10% gel) and then transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). Subsequent to blocking by 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA), membranes were incubated with the following primary antibodies at 4˚C overnight: Rabbit monoclonal Lin28B antibody diluted at 1:1,000 (Abcam) and polyclonal rabbit anti-GAPDH antibody diluted at 1:5,000 (ab115698; Abcam). The polyvinylidene fluoride membranes were then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (1:4,000; ab6721; Abcam) for 1 h after washing with PBST (Phosphate Buffered Saline containing 1% Tween-20). Finally, proteins were visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.).

Statistical analysis. All data are expressed as the mean ± standard deviation. Student's t-test was used to compare the significance of differences between two or more groups, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

High expression of Lin28B was detected in colon cancer tissues. The expression pattern of Lin28B was investigated in colon cancer. Using immunohistochemistry, the expression of Lin28B was detected in 92 colon cancer tissues and 11 adjacent colon tissues. The results indicated that the Lin28B protein was expressed in all colon cancer tissues, resulting in a positive rate of Lin28B expression of 100%, while only ~9.1% of adjacent colon tissues expressed Lin28B (1/11) (Fig. 1). As shown in Fig. 1, it was found that Lin28B was mainly expressed in the cytoplasm of cancer cells, which is contrary to previous observation (3). It was reported

Table II. Association between clinical characteristics and Lin-28 homolog B expression.

Clinical parameters	Patients, n	Immunohistochemistry score[a]	P-value
Age (range, 39-75)			>0.05
≤50 years	39	5.3±1.4	
>50 years	53	4.8±2.6	
Sex			>0.05
Male	63	5.7±3.4	
Female	29	3.7±2.7	
Morphology			>0.05
Ulcerated	15	4.7±2.4	
Polypous	73	3.8±2.3	
Infiltrating	4	4.3±1.8	
Differentiation			>0.05
Well	21	5.4±3.4	
Moderate	49	4.3±1.7	
Poor	22	5.1±2.7	
Lymph node metastasis			>0.05
Yes	33	5.7±4.4	
No	59	4.3±2.3	
Tumor size			>0.05
≤5 cm	43	4.9±2.5	
>5 cm	49	5.2±3.7	

[a]Data are presented as the mean ± standard deviation.

that Lin28B not only promotes the proliferation, but also facilitates the metastasis of cancer cells (4). The association between Lin28B expression and clinical parameters of patients with colon cancer was then analyzed. As shown in Table II, the expression level of Lin28B was not associated with any clinical parameter. Thus, Lin28B was not observed to have an impact on the proliferation and metastasis of colon cancer in the present study.

Overexpression of Lin28B increased chemotherapeutic sensitivity of colon cancer cells to 5-FU. Since no association was identified between Lin28B expression and tumor size or lymph node metastasis, the impact of Lin28B on the chemotherapeutic sensitivity of colon cancer cells was then detected. To identify the potential role of Lin28B in chemotherapy of colon cancer, a Lin28B over-expressing HCT116 (EGFP-Lin28B-HCT116) cell line was established. In accordance with the immunohistochemistry results, Lin28B-GFP expression was predominantly distributed in the cytoplasm (Fig. 2A), and the overexpression of Lin28B was confirmed using quantitative PCR (Fig. 2B) and western blot analysis (Fig. 2C). The control EGFP-HCT116 and Lin28B over-expressing EGFP-Lin28B-HCT116 cells were then exposed to various concentrations of 5-FU for 48 h. A luminescence-based cell viability assay was used to detect the effect of Lin28B on the chemotherapeutic sensitivity of colon cancer cells. The result showed that, compared with the control cells, the sensitivity of cells with Lin28B overexpression to 5-FU is significantly increased ($P<0.01$; Fig. 2D).

Lin28B overexpression promoted the cell apoptosis induced by 5-FU. To detect the potential mechanism by which Lin28B enhances the chemosensitivity of colon cancer cells to 5-FU, the present study evaluated the effect of Lin28B overexpression on the apoptosis of HCT116 cells induced by 5-FU. The present results showed that the apoptosis rate of EGFP-Lin28B-HCT116 cells was significantly increased compared with the EGFP-HCT116 cells (student's t-test, $P<0.05$, Fig. 3). This result suggested that Lin28B may increase the chemosensitivity of colon cancer cells to 5-FU by enhancing cell apoptosis.

Lin28B increased the chemosensitivity of colon cancer cells to 5-FU in a let-7 independent manner. It is well known that let-7 is one of the most frequently studied targets of Lin28B. To identify the molecular mechanism by which Lin28B increases the chemosensitivity of colon cancer cells to 5-FU, the potential involvement of let-7 was then evaluated. As expected, the overexpression of Lin28B in HCT116 cells significantly inhibited the expression of let-7 (Fig. 4A), which is consistent with previous studies (14). Subsequently, the effect of let-7 on colon cancer chemotherapy was investigated. It was shown that the overexpression of let-7b in HCT116 cells significantly enhanced the chemosensitivity of colon cancer cells to 5-FU ($P<0.01$; Fig. 4B). This result is same as the effect of Lin28B on the chemotherapy of colon cancer treatment with 5-FU, even though the overexpression of Lin28B significantly inhibits the expression of let-7. Overall, these results indicated that Lin28B increased the chemosensitivity of colon cancer cells to 5-FU in a let-7 independent manner.

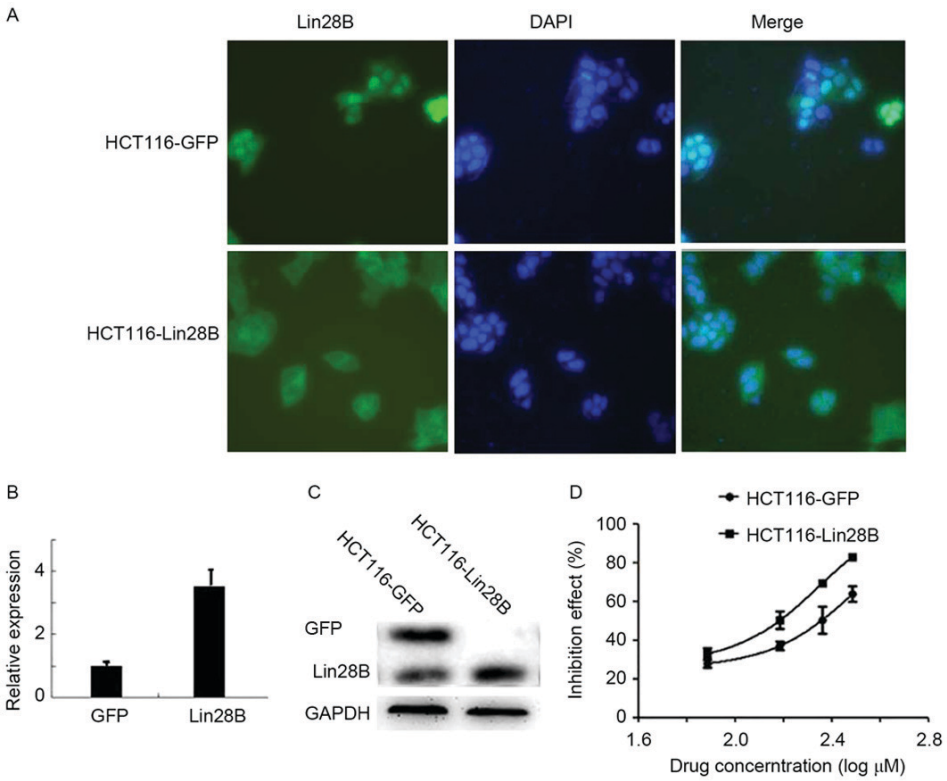

Figure 2. Overexpression of Lin28B promotes the chemotherapeutic sensitivity of colon cancer cells to 5-FU. (A) Lin28B was predominantly located in the cytoplasm of HCT116 cells, as illustrated by GFP distribution, and the nucleus was stained by DAPI (magnification, x200). (B) Overexpression of Lin28B was confirmed by quantitative polymerase chain reaction. (C) Overexpression of Lin28B was confirmed by western blot analysis. (D) The overexpression of Lin28B significantly increased the chemosensitivity of HCT116 cells treated with 5-FU ($P<0.05$). Lin28B, Lin-28 homolog B; 5-FU, 5-fluorouracil; GFP, green fluorescent protein.

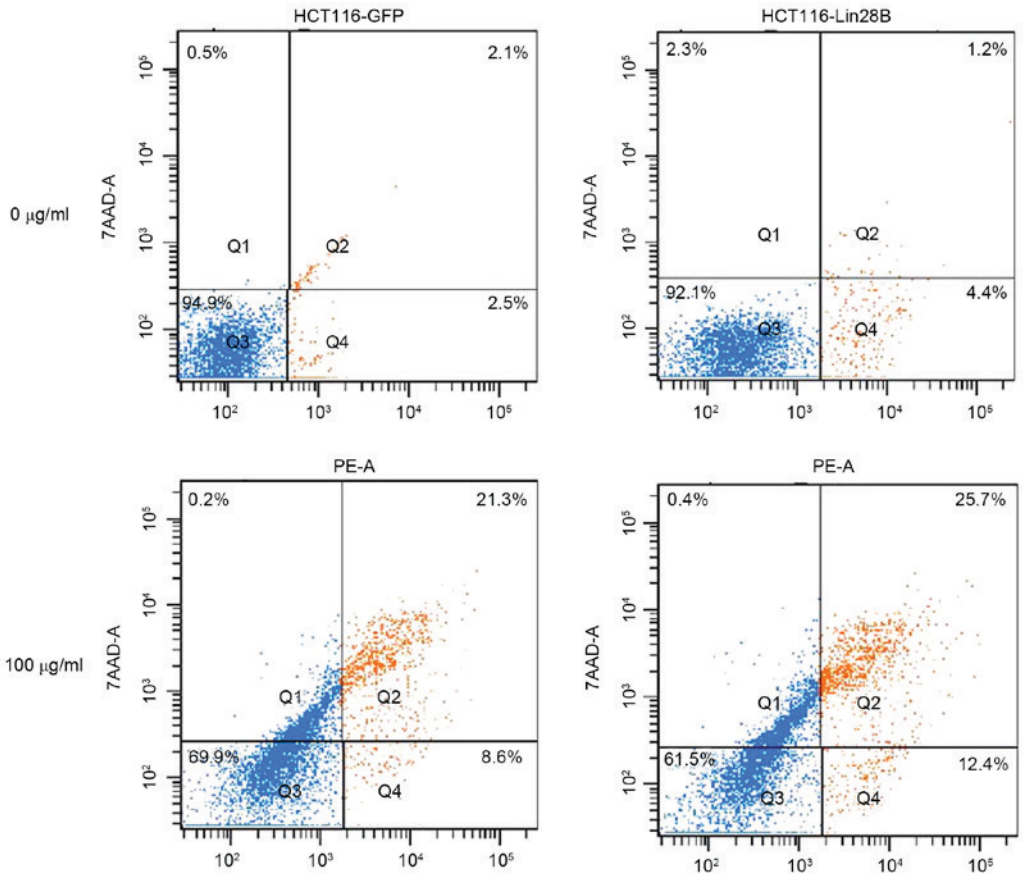

Figure 3. Lin28B overexpression promoted the apoptosis of HCT116 cells induced by 5-FU. Lin28B, Lin-28 homolog B.

Discussion

The RNA-binding Lin28 protein family consists of two members, Lin28A and Lin28B. Lin28A was initially known as a developmental timing regulator and functions as a stemness maintenance factor, whereas Lin28B was first identified in hepatocellular carcinoma (4,15,16). Both proteins have been demonstrated to block the biogenesis of microRNA let-7s by interacting with primary or precursor let-7s and preventing their maturation or inducing their degradation (3). Previous studies have demonstrated that Lin28A and Lin28B are each frequently upregulated in various malignancies, and the overexpression of Lin28A and Lin28B is associated with multiple biological behaviors of cancers and poor prognosis (6,17). However, few studies have focused on the role of Lin28A or Lin28B in cancer chemotherapy.

The present study showed that Lin28B was upregulated in all assessed colon cancer tissues. Differing from a previous study (3), it was found that Lin28B was mainly expressed in the cytoplasm instead of the nucleus of colon cancer cells. To the best of our knowledge, the present study is the first to report the cytoplasmic distribution of Lin28B in colon cancer cells. Since a previous study demonstrated that Lin28B expression is associated with an advanced stage of malignant tumors (18), the present study hypothesized that the elevated expression of Lin28B in colon cancer may also act as an oncogene. However, the association analysis between Lin28B expression and clinical characteristics indicated that Lin28B expression level is not associated with tumor growth and metastasis. The function of Lin28B in the chemosensitivity of colon cancer cells was then evaluated. Notably, it was identified that the overexpression of the Lin28B gene, which has oncogenic potential, enhanced the sensitivity of colon cancer HCT116 cells to 5-FU. Additional investigation showed that enforced expression of Lin28B promoted the apoptosis of colon cancer cells induced by 5-FU. The present results are consistent with a previous study by Teng *et al* (19), who revealed that Lin28B expression was significantly increased in gastric cancer patients with pathological response to neoadjuvant chemotherapy, which indicated that Lin28B expression is associated with the sensitivity of neoadjuvant chemotherapy in gastric cancer. However, the effect of Lin28 on chemotherapy in other types of cancer may vary. The promotion of resistance of breast cancer cells to paclitaxel by overexpression of Lin28 has been reported, and the induction of p21 and Rb expression and inhibition of let-7a miRNA generation contributes to Lin28-mediated resistance to chemotherapy in breast cancer (20). Additionally, Lin28B-inducedpromotion of chemoresistance to paclitaxel was also reported in hepatocellular carcinoma (21). Investigation of the mechanism revealed that inhibition of let-7 production and the consequent upregulation of its target B-cell lymphoma-extra large underlined the paclitaxel resistance in HCC cells (21). The role of Lin28B in the chemosensitivity of colon cancer cells in the present study differs from the observations in these previous studies. The mechanism of the opposing roles of Lin28B in chemotherapy in different cancer types requires additional experiments to evaluate.

Let-7 is a well-known target of Lin28B, and based on current studies, the majority of the reported functions of Lin28B are associated with the repression of let-7 production (6). By binding either pri-let-7 or pre-let-7 and blocking their processing by Drosha or Dicer, or by recruiting TUT4/TUT7 to oligo-uridylate the of pre-let-7, Lin28B negatively regulates the generation of let-7 miRNAs (22-27). In the present study, it was also shown that the over-expression of Lin28B in colon cancer cells significantly attenuated the let-7 level, which is consistent with previous studies (3,4,8,9). To detect the molecular mechanism of Lin28B in promoting chemosensitivity of colon cancer cells to 5-FU, the effect of let-7 in the chemosensitivity of colon cancer cell to 5-FU was also evaluated. The results showed that the overexpression of let-7b significantly increased the chemosensitivity of colon cancer cells to 5-FU, which indicated that the Lin28B-induced promotion of chemosensitivity of chemosensitivity of colon cancer cells to 5-FUis independent of let-7. The mechanism of this effect of Lin28B on the chemosensitivity of cells requires additional investigation.

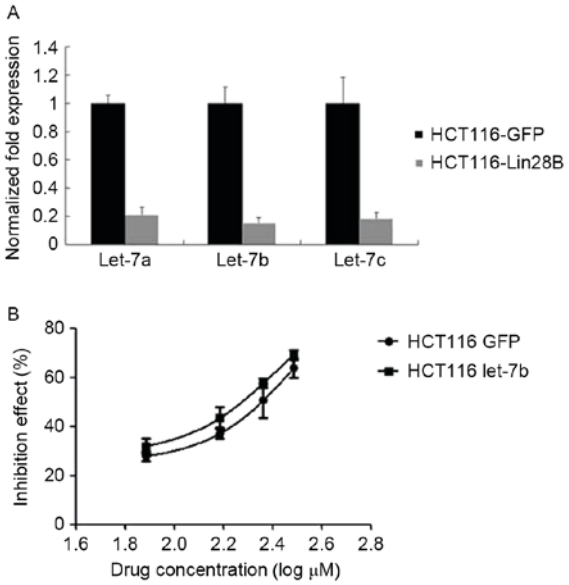

Figure 4. Lin28B increased the chemosensitivity of HCT116 cells to 5-FU in a let-7-independent manner. (A) The overexpression of Lin28B in HCT116 cells repressed the expression of let-7a, let-7b and let-7c, as determined by quantitative polymerase chain reaction. (B) The overexpression of let-7 significantly enhanced the chemosensitivity of HCT116 cells to 5-FU ($P<0.05$). Lin28B, Lin-28 homolog B; 5-FU, 5-fluorouracil; GFP, green fluorescent protein.

In conclusion, the present study showed that overexpression of the oncogene Lin28B increased the chemosensitivity of colon cancer cells to 5-FU by promoting cell apoptosis. Additional investigation indicated that this effect of Lin28B-induced enhancement of chemosensitivity was let-7-independent. The present results indicated that Lin28B may act as a predictive biomarker for chemotherapy in patients with colon cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LM and YZ conceived and designed experiments. LM, QZ and WC conducted the experiments and collected data. LM and QZ analyzed data. LM wrote the manuscript and was responsible for manuscript revisions and YZ assisted with constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Tumor Hospital Affiliated to Harbin Medical University. All participants provided written informed consent.

Consent for publication

All study participants provided informed consent for the publication of this data.

Competing interests

The authors declare no conflict of interest.

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