LIN28B Promotes Colon Cancer Migration and Recurrence

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Abstract
LIN28B is involved in “stemness” and tumourigenesis by negatively regulating the maturation of let-7 microRNA family members. In this study, we showed that LIN28B expression promotes migration and recurrence of colon cancer. Immunohistochemistry and reverse-transcription polymerase chain reactions were performed to detect LIN28B expression in colon cancer tissue microarrays, paraffin-embedded surgical resected tissues and cancer cells. Loss-of-function, migration and proliferation analyses were performed to delineate the potential roles of LIN28B in colon cancer. LIN28B was upregulated in colon cancer tissue compared to normal mucosa, and its overexpression correlated with reduced patient survival and increased tumour recurrence. LIN28B suppression inhibited the migration of SW480 colon cancer cells and facilitated the cytotoxicity induced by oxaliplatin in SW480 and HCT116 colon cancer cells. In conclusion, LIN28B overexpression contributes to colon tumourigenesis, and LIN28B may serve as a diagnostic tool and therapeutic target for colon cancer.

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Introduction
LIN28 was initially identified in C. elegans and was shown to be responsible for the timing of development [1]. LIN28 induces pluripotency when expressed in somatic fibroblasts along with OCT4, SOX2 and KLF4. LIN28B, as a homologue of LIN28, was first cloned from and shown to be overexpressed in human hepatocellular carcinoma cells and clinical samples in 2006 [2]. LIN28B is a developmentally regulated RNA-binding protein that promotes tumourigenesis by blocking the post-transcriptional processing of the tumour-suppressive pri-/pre-let-7 microRNA [3,4]. The ability of LIN28B to regulate let-7, a bona fide tumour-suppressor, is consistent with its developmental role in regulating cell proliferation and differentiation. Interestingly, several studies revealed that LIN28B itself is also a target of let-7 family members, including miR-125b [5]. Besides let-7, LIN28B may also bind the mRNA of intestinal stem cell markers, such as LGR5 and PROM1 [6]. In summary, LIN28B promotes transformation primarily by suppressing let-7. Competition between LIN28B and let-7 may be critical for normal cell biology and may accelerate tumour development once this balance is perturbed.

LIN28B is hypermethylated in somatic tissues [7], and its aberrant reactivation may promote tumourigenesis [8]. LIN28B is frequently overexpressed in multiple cancers, especially advanced types such as progressive hepatocellular carcinoma [2,9], epithelial ovarian cancer [10], Wilms’ tumour and germ cell tumours [9]. In addition to being targeted by antecedent microRNAs, LIN28B is activated by several oncogenic pathways. C-/N-Myc induce LIN28B expression in multiple human and mouse tumour models, resulting in let-7 repression and cell proliferation [11,12]. MYCN amplification also results in LIN28B overexpression [10], and it has been reported that c-Myc is related to sporadic large bowel cancer and familial polyposis coli [13], implying a role for LIN28B in colon cancer.

Despite great achievements in surgery, chemotherapy and the development of novel molecular-targeted drugs, such as bevacinumab (Avastin), the incidence of colon cancer continues to increase [14]. Each year, tumours of the colon are responsible for 655,000 deaths globally. Because Let-7 functions as a potential growth suppressor in human colon cancer cells [15], we examined LIN28B expression in colon cancer tissues to determine the balance between LIN28B and let-7 expression. We examined LIN28B expression in human colon cancer tumours via tissue microarray and found that LIN28B was significantly upregulated in tumour tissue compared to normal colonic mucosa. To further analyse the survival of patients with colon cancer, we selected an additional cohort of postoperative patients with detailed pathology records and follow-up data from 2004 to 2009. As anticipated, LIN28B overexpression correlated with reduced patient survival and an increased likelihood of tumour recurrence. Furthermore, we found that silencing LIN28B inhibited the migration of SW480...
cells and sensitised SW480 and HCT116 colon cancer cells to oxaliplatin-induced cytotoxicity.

**Patients and Methods**

**Tumour tissue analysis**

Samples of human colon carcinomas and normal colons were obtained from a human colon carcinoma tissue array (CO2161; US Biomax), which contained 204 adenocarcinomas, 4 signet ring cell cancers and 8 normal colon mucosa samples. Detailed clinical information, including differentiation status and TNM grading, were also provided for each sample. An additional cohort of 149 colon carcinoma samples was collected from specimens that had been surgically resected in 2004 from consenting patients at Nanfang Hospital (Southern Medical University, Guangzhou, China), according to an Institutional Review Board–approved protocol. Each of these cases was followed, in detail, through June 2009. We confirmed the TNM grade for samples from both cohorts. In total, 357 colon cancer tumours and 8 normal colon samples were used for the IHC analysis. The detailed information for all tumours and patients is provided in Table 1. The study protocol was approved by the ethics review board of Nanfang Hospital. We have obtained written informed consent from all study participants. All of the procedures were done in accordance with the Declaration of Helsinki and relevant policies in China.

Two pathologists scored the LIN28B staining intensity according to the following scale: a score of 0/1 was used to signify weak/low LIN28B intensity (0–25% positive rate); a score of 2 represented intermediate intensity (25–50% positive rate); and a score of 3 was used for high-intensity staining (>50% positive rate). Tumours of grades 1, 2 and 3 are equivalent to tumours classified as well differentiated, moderately differentiated or poorly differentiated, respectively, using microscopy.

Log rank tests (Mantel–Cox and Breslow) were performed to compare the survival and recurrence distributions of the different intensity groups (0–2 vs. 3). The correlation between the staining intensity and survival or recurrence was determined using a chi-square analysis, and a 95% confidence interval was calculated to determine statistical significance.

**Cell culture**

The SW480, Caco2 and HCT116 colon cancer cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA) and were cultured in Dulbecco’s modification of Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS; HyClone, South Logan, UT, USA). All cell lines were maintained in a 5% CO₂, humidified atmosphere.

**Oligoribonucleotides**

A LIN28B-specific siRNA and a negative control small RNA (NC) were constructed by GenePharma (Shanghai, China). LIN28B- and GAPDH-specific primers were synthesised by TaKaRa (TaKaRa Biotechnology, Dalian, China).

**Cell transfection**

RNA oligoribonucleotides were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Between 50 and 100 nM of the RNA oligoribonucleotides were used for each transfection.

**Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using TRIzol reagent (Invitrogen) and was then treated with DNase I (Tiangen Biotech, Beijing, China). The cDNA for the qRT-PCR was synthesised with the PrimeScript RT reagent kit and the gDNA Eraser (TaKaRa). The PCR amplification procedure for GAPDH was as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; and a terminal elongation at 72°C for 5 min. The amplification of LIN28B was performed as follows: 95°C for 2 min; 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s; and a final step at 72°C for 5 min. The qRT-PCR assays were performed using the SYBR PrimeScript RT-PCR kit (TaKaRa) for GAPDH and LIN28B.

**Table 1. Clinical characteristics of patients/tumours at the time of diagnosis.**

| Clinical variants | Tissue microarray (n = 208) | Surgically resected tumours (n = 149) |
|------------------|-----------------------------|--------------------------------------|
| Average age (years) | 54.80 | 56.9 |
| Sex              |                              |                                      |
| male             | 118                          | 88                                   |
| female           | 90                           | 61                                   |
| Pathology grade  |                              |                                      |
| moderately/well-differentiated | 150   | 137                                |
| poorly differentiated | 41     | 12                                  |
| TNM grade        |                              |                                      |
| I                | 22                           | 26 (1 Tis)                           |
| II               | 128                          | 65                                   |
| III              | 47                           | 42                                   |
| IV               | 11                           | 16                                   |
| Tumour type      |                              |                                      |
| adenocarcinomas  | 204                          | 148                                  |
| signet ring cell cancers | 4     | 1                                   |

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Immunoblots
Cytosolic protein fractions were prepared using RIPA buffer (Beyotime Biotechnology, Haimen, China). The antibodies used for the immunoblots were specific for LIN28B (1:10, ab71415; Abcam, Cambridge, MA, USA) and GAPDH (G9295; Sigma-Aldrich, USA). Western blots and IHC were performed according to standard procedures.

Migration analysis
SW480 cells (1×10⁴ cells in 100 μL serum-free medium), which had been transfected with the NC or si-LIN28B, were placed in the top chamber of Transwell culture dishes (8 μm; BD Biosciences, San Jose, CA). The lower chamber was filled with 600 μL of conditioned medium. After 24 hours, the cells that had not migrated to the lower chamber were removed from the upper surface of the Transwell membrane with a cotton swab. Migrated cells on the lower membrane surface were fixed, stained with 0.1% crystal violet and imaged.

Cell viability analysis
SW480 or HCT116 cells, which had been transfected with either the NC or si-LIN28B, were seeded in triplicate in 96-well plates at a concentration of 1×10⁴ cells per well. After the cells had adhered to the plates, they were incubated with different

Figure 1. LIN28B is significantly overexpressed in colon tumour tissues. IHC showed that LIN28B was markedly upregulated in tumour tissues compared with the normal mucosa, which demonstrated very little LIN28B expression. Representative graphs are presented.
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Figure 2. LIN28B overexpression correlated with reduced patient survival and an increased likelihood of tumour recurrence. (A) Higher LIN28B staining intensity from stage I, II and III colon cancers correlated with reduced patient survival. (B) High LIN28B expression was related to a higher probability of tumour recurrence (p<0.01; log rank test).
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concentrations of oxaliplatin (e.g., 100, 10, 1, 0.1, 0.01 and 0.001 mg/mL; Jiangsu Hengrui Medicine Co., Ltd., Jiangsu, China) for 4 hours and were then transferred to complete medium for an additional 20 hours. At the indicated time point, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well, and the cells were incubated for 3 h at 37°C. The absorbance (A) was recorded at 450 nm using a plate reader. The experiment was performed in triplicate, and the cell inhibition ratio was determined using the following equation: (1 - test group A/control group A) × 100%. The IC50 (50% inhibiting concentration) value was calculated using specific software (LOGIT method).

Statistical analysis

A log rank test was performed to compare the survival and recurrence distributions for the various intensity groups (0–2 vs. 3). The correlation between staining intensity and survival or recurrence was determined according to a chi-square analysis, where p values less than 0.05 were considered to be statistically significant. A 95% confidence interval was calculated for confirmation of statistical significance.

Results

LIN28B was upregulated in colon carcinomas

To examine the potential roles for LIN28B in colon cancer, we first compared LIN28B protein expression between 208 tumour samples and 8 non-matched normal mucosa samples from a tissue microarray using IHC. The staining intensity of LIN28B was scored by two pathologists who were blinded to the clinical information. Consistent with previous reports [6,16], LIN28B was significantly overexpressed in tumour tissues compared to normal tissues (Fig. 1, p<0.001).

LIN28B overexpression correlated with reduced patient survival and a high risk of recurrence

Data from patients who underwent surgery (TNM I-III, paraffin-embedded surgical resected tissues) were further analysed via log rank tests to investigate the influence of LIN28B overexpression on patient survival and recurrence. This analysis revealed a correlation between low-intensity LIN28B staining from samples of TNM grade I and II tumours and increased patient survival (Mantel-Cox p<0.01; Breslow, p<0.01) and a lower likelihood of tumour recurrence (Mantel-Cox p<0.01; Breslow p<0.01) (Fig. 2).

Taken together, our results reveal that LIN28B expression is closely related to overall patient survival and recurrence of colon cancer.

LIN28B loss-of-function sensitised SW480 and HCT116 cells to oxaliplatin-mediated cytotoxicity

The let-7 tumour suppressor microRNA family members are known to regulate chemosensitivity [17], while LIN28B promotes malignancy mainly by inhibiting let-7 biogenesis. Thus, we sought to determine whether LIN28B could influence chemosensitivity to oxaliplatin. We examined LIN28B expression in three colon cancer cell lines using RT-PCR and Western blotting (Fig. 3A). HCT116 and SW480 cells were selected for use in these
experiments due to their low and high level of Lin28 expression, respectively. Oxaliplatin induced concentration-dependent cytotoxicity in these cells (Fig. 3B). The IC_{50} value for oxaliplatin was 6.23±0.75 μg/mL for the HCT116 cells, and this was increased by 30% to 10.7±2.26 μg/mL in the SW480 cells. This finding suggested that differences in LIN28B expression may serve to modulate chemosensitivity in colon cancer cells. To verify this hypothesis, we constructed LIN28B-specific siRNA and control small RNA. The efficiency of the si-LIN28B was determined in both cell lines (Fig. 4A–D), and then a CCK-8 analysis was performed to explore the interactions between the si-LIN28B and oxaliplatin in HCT116 and SW480 cells. The results indicated a synergistic effect between si-LIN28B and oxaliplatin (Fig. 3C–D), which suggests that the targeting of LIN28B may be capable of sensitising colon cancer cells to oxaliplatin therapy.

Downregulation of LIN28B repressed the migration of SW480 cells

As LIN28B overexpression was correlated with tumour recurrence and patient survival, we next sought to explore whether LIN28B expression influences the migration of colon cancer cells. We knocked down LIN28B expression using siRNA, and the Transwell analysis indicated that suppression of LIN28B significantly inhibited the migration of SW480 cells (Fig. 5).

Discussion

Several stemness-related genes, which are referred to as oncofoetal genes, are thought to promote tumourigenesis upon re-expression in somatic cells. The presence of CD133⁺ cancer stem cells or cancer-initiating cells may explain the metastasis, recurrence and chemo-resistance of colon cancer [18,19]. Let-7 is involved in regulating the self renewal and tumourigenicity of breast cancer-initiating cells [20] and is also repressed in colon cancer cells [15]. LIN28B overexpression is known to contribute to carcinogenesis by blocking the biogenesis of let-7, which prompted us to evaluate whether the balance between tumour-promoting LIN28B and tumour-suppressive let-7 was altered in colon cancer...
that the LIN28B-let-7-c-Myc/LIN28B
our findings reveal the existence of a preliminary feedback loop accelerates let-7 pathway in colon carcinogenesis. We hypothesise that Wnt/APC-Myc/LIN28B-let-7 pathway activation is the initiating event and that the LIN28B-let-7-c-Myc/LIN28B feedback loop accelerates colon carcinogenesis. However, further studies are required to verify the role of this feedback loop in carcinogenesis.

Metastasis and recurrence are the primary reasons for the decrease in overall survival among patients with malignant tumours. Thus, it is of great interest to be able to predict tumour metastasis and recurrence at an early stage. Furthermore, predicting tumour recurrence for stage II/III patients may guide adjuvant chemotherapy. Improved methods for identifying stage II patients at a high risk of recurrence [26], which are based on the unique characteristics of each individual tumour, may result in thousands of lives saved each year. In addition, techniques for identifying stage III patients who are at a low risk for disease recurrence after surgery may spare these individuals from the cost, time and toxicity associated with chemotherapy [27]. In our study, LIN28B staining intensity (≥50%) correlated with reduced patient survival. Importantly, LIN28B expression could predict the recurrence of TNM grade II/III tumours after surgical resection.

While this manuscript was in preparation, a study from King et al. reported that LIN28B promotes colon cancer progression and metastasis both in vivo [16] and in vitro through let-7-dependent and -independent mechanisms [6]. Our data are consistent with these findings, and we also suggest that detecting the expression of LIN28B might aid in determining the appropriate scheme of adjuvant chemotherapy in patients with TNM grade II and III colon carcinomas.

In conclusion, our data suggest an important role for LIN28B in colon carcinogenesis. LIN28B expression was shown to predict patient survival, which implies that LIN28B may be a useful target for novel molecular therapeutics.

Author Contributions
Conceived and designed the experiments: MP KW. Performed the experiments: MP GW XH NH. Analyzed the data: LL GJ PS. Contributed reagents/materials/analysis tools: BL GL. Wrote the paper: MP.