LMNB2 Promotes the Progression of Colorectal Cancer by Silencing p21 Expression

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Abstract

Background: Lamin B2 (LMNB2) is involved in chromatin remodelling and the rupture and reorganization of the nuclear membrane during mitosis, which is necessary for eukaryotic cell proliferation. However, there are few reports on the expression and function of LMNB2 in colorectal cancer.

Methods: A tissue microarray (TAM) was used to detect the expression of LMNB2 in 226 colorectal cancer tissues and the corresponding adjacent tissues. The CCK-8 colorimetric assay, EdU incorporation analyses, colony formation assays and cell cycle experiments were used to evaluate the effect of LMNB2 on colorectal cancer cell proliferation in vitro, and a mouse tumorigenic model was used to study the effect of LMNB2 on colorectal cancer cells in vivo. The main pathways and genes regulated by LMNB2 were detected by RNA sequencing. Dual-luciferase reporter assays were conducted to test the direct binding between LMNB2 and p21, and ChIP analysis showed that LMNB2 promotes cell proliferation by regulating the p21 promoter.

Results: The results showed that LMNB2 expression is increased in colorectal cancer tissues. Highly expressed LMNB2 is associated with tumour size and TNM stage. Multivariate Cox analysis showed that LMNB2 can be used as an independent prognostic factor in patients with colorectal cancer. Functional assays indicated that LMNB2 obviously enhanced cell proliferation by promoting cell cycle progression in vitro and in vivo. LMNB2 facilitates cell proliferation via regulating the p21 promoter, whereas LMNB2 had no effect on cell apoptosis in terms of mechanism.

Conclusion: LMNB2 promotes the proliferation of colorectal cancer by regulating p21-mediated cell cycle progression, indicating the potential value of LMNB2 as a clinical prognostic marker and molecular therapeutic target.

Background

Colorectal cancer (CRC) is one of the most common malignant tumours worldwide. According to the latest data, the incidence of colorectal cancer ranks third in the incidence of malignant tumours, and the mortality rate ranks second(1). In recent years, due to early detection and advanced treatment, colorectal cancer mortality has declined(2). However, the complex developmental mechanism of colorectal cancer still hinders the treatment of this disease. Therefore, more detailed mechanisms and reliable markers are needed to predict the survival of colorectal cancer patients.

Lamins are components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane, which is thought to provide a framework for the nuclear envelope(3). The lamin family includes lamin A, lamin C, lamin B1, lamin B2, and lamin B3. The C-terminus is composed of a highly conserved layer tail domain and is involved in protein-protein interactions and protein-nucleus interactions(4–7). Lamin proteins are thought to be involved in nuclear stability, chromatin structure and gene expression(8). The lamins also have other functions, such as playing a role in chromatin organization, connecting the nucleus and cytoplasm, regulating gene transcription, and mitosis(9–11).

Studies have shown that lamin A also regulates gene expression by binding chromatin to the periphery of the nucleus(12). Lamin A/C is involved in gene expression related to cell cycle regulation(13). Lamin A/C is overexpressed in neuroblastoma(14), prostate cancer(15), hepatocellular carcinoma(16), breast cancer(17) and low-grade endometrial cancer(18). Given the role of lamins in these processes, dysregulation of lamin expression and function will lead to cell death, abnormalities and tumorigenesis.

Lamin B2 (LMNB2) is an important member of the lamin protein family. The human LMNB2 gene is located on chromosome 19p13.3(19) and encodes a 68 kDa protein consisting of 660 amino acids. Its main function is to maintain the integrity of the nuclear skeleton and participate in cell proliferation and ageing, gene expression and DNA damage repair by affecting chromosome distribution(20, 21). Lamin B2 is overexpressed in hepatocellular carcinoma(22). Mutations in the LMNB2 gene cause progressive myoclonic epilepsy with early ataxia(20, 23). Lamin B2 is overexpressed in ovarian cancer. In addition, LMNB2 can promote the proliferation and tumour formation of non-small cell lung cancer(24, 25). This study is the first to report the effect of LMNB2 on cell proliferation and metastasis in CRC and to explain the molecular mechanism of LMNB2 in CRC cell proliferation.

In this study, to evaluate the role of LMNB2 in colorectal cancer, we used a tissue microarray (TMA) combined with a retrospective cohort of colorectal cancer patients to study the relationship between LMNB2 protein expression in colorectal cancer and clinicopathological characteristics. In addition, we investigated whether LMNB2 can be used as an independent prognostic biomarker for targeted therapy in patients with colorectal cancer. We showed that LMNB2 promotes the proliferation of colorectal cancer cells in vitro and in vivo through transcriptional regulation of p21. This study reported for the first time the effect of LMNB2 on the proliferation of colorectal cancer cells and explained the molecular mechanism of LMNB2 in the proliferation of colorectal cancer. These data provide new insights into the pathogenesis of colorectal cancer and support the potential value of LMNB2 as a treatment target for this disease.

Methods

Patient and specimens

The tissue chip (TMA) is composed of 226 colorectal cancer tissues and matched adjacent tissues collected by the Department of Pathology, Affiliated Hospital of Xuzhou Medical University, all of which are paraffin embedded blocks. All patients underwent radical surgery in the Affiliated Hospital of Xuzhou Medical University from April 2010 to March 2015. Clinicopathological characteristics such as the patient's age, sex, lymph node metastasis, tumour-node-metastasis (TNM) stage, degree of differentiation, tumour diameter, distant metastasis and infiltration depth were obtained from the Department of Pathology, Affiliated Hospital of Xuzhou Medical University. A total of 8 pairs of fresh colorectal cancer tissues and adjacent tissues were taken directly from the operating room of the Affiliated Hospital of Xuzhou Medical University, and postoperative pathology confirmed colorectal adenocarcinoma. The clinical pathological information of the patients was obtained from the hospital medical records, and informed consent was obtained from all patients. The patient study was conducted

in accordance with the Declaration of Helsinki. The use of these specimens and data for research purposes was approved by the hospital ethics committee. Clinicopathological characteristics such as the patient's age, sex, lymph node metastasis, tumour-node-metastasis (TNM) stage, degree of differentiation, tumour diameter, distant metastasis and infiltration depth were obtained from the Department of Pathology, Affiliated Hospital of Xuzhou Medical University. A total of 8 pairs of fresh colorectal cancer tissues and adjacent tissues were taken directly from the operating room of the Affiliated Hospital of Xuzhou Medical University.

Immunohistochemistry (IHC)

The paraffin-embedded CRC tissues and corresponding paracancerous tissues were punched to obtain 1.5 mm diameter cores. The standard protocol for immunostaining of the TMAs was described previously. Immunohistochemistry was performed according to the streptavidin–peroxidase (Sp) method using a standard Sp Kit (Zhongshan Biotech, Beijing, China). The TMA slide was incubated with monoclonal rabbit anti-LMNB2 (1:100, ab151735, Abcam, Cambridge, MA, USA) overnight at 4 °C, anti-p21 antibodies were applied at 1:100 dilutions (#2947, Cell Signaling Technology, USA), and anti-Ki67 antibodies were applied at 1:200 dilutions (ab16667, Abcam, USA). Diaminobenzidine (DAB; Zhongshan Biotech, Beijing, China) was used to produce a brown precipitate. The immunoreactivity was assessed blindly by two independent observers using light microscopy (Olympus BX-51), and the image was collected by a Camedia Master C-3040 digital camera (Beijing, China).

Assessment of IHC

Two pathologists separately assessed the TMAs under blinded experimental conditions, and all differences that arose were resolved by discussion. The staining scores of LMNB2 were evaluated by combining the percentage of cells with the staining intensity and were dependent on the IRS. The intensity of LMNB2 immunostaining was scored as 0–3 (0, negative; 1, weak; 2, moderate; 3, strong); the percentage of immunoreactive cells was graded as 1 (0–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). Based on the IRS, the level of LMNB2 expression was categorized as low (IRS: 0–6) and high (IRS: 8–12) expression.

Cell lines and cell culture

The colorectal cancer cell lines FHC, HCT116, DLD1, LOVO, HCT29, SW480, and SW620 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai China). HCT116, SW480, and SW620 cells were cultured in DMEM, while DLD1 and LoVo cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum and 100 µg/ml streptomycin and incubated in a 37 °C humidified incubator with 5% CO₂.

Small interfering RNA and transient transfections

Small interfering RNA (siRNA) specific for LMNB2 (siLMNB2) and nonspecific control siRNA (siCtrl) were purchased from GenePharma (Shanghai, China) and transfected with siLentFect Lipid Reagent (Bio-Rad

Laboratories, Inc.) according to the manufacturer's protocol when CRC cells were grown to ~50% confluency. Six hours after transfection, the medium containing transfection reagents was replaced by fresh medium. The siRNA sequences are as follows:

SiLMNB2#1 sense: GCGAGGUGAGUGGCAUCAATT;

SiLMNB2#2 sense: GGAAGUGGCCAUGAGGACUTT;

sip21# sense: CCUCUGCAUUAGAAUUAUTT;

siCtrl sense: UUCUCCGAACGUGUCACGUTT.

Stable cell line generation

For stable suppression of LMNB2 expression, LMNB2 short hairpin RNA (shRNA) and control lentivirus were obtained from GenePharma. HCT116 and DLD1 cells were infected with lentivirus for 48 h and then selected with 2 ng/ml puromycin for 2 weeks, with the medium refreshed every 3 days. The shRNA target sequences were as follows: shLMNB2 sense: GCGAGGUUGGCAUCAATT;

shCtrl sense: TTCTCCGAACGTGTACGT.

Cell proliferation and colony formation assays

CCK-8 assays were carried out to determine the function of LMNB2 in cell proliferation. HCT116 and DLD1 cells were transfected with siRNA targeting LMNB2 or negative control siRNA using siLentFect™ Lipid Reagent for RNAi (Bio-Rad Laboratories, Inc.), respectively. Forty-eight hours after transfection, for CCK-8 analysis, 4000 cells were seeded in each well of 96-well plates, and CCK-8 solution was added 24, 48, 72, and 96 h after transfection. Cells were incubated at 37 °C for 1 h after 10 µl of CCK-8 solution was added. The absorbance at 450 nm was measured. Five millilitres of cell suspension containing 7×10^2 cells was inoculated into a 60 mm dish for continuous culture until visible clones appeared. Then, the cells were fixed with methanol and stained with 0.05% crystal violet solution. After two washes with PBS, the plates were photographed using a digital camera. Positive colony formation, defined as colonies with more than 50 cells, was confirmed by manual counting.

EdU assay

The effect of LMNB2 on the proliferation of HCT116 and DLD1 cells was measured by a 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay using an EdU assay kit (RiboBio, Guangzhou, China) according to the manufacturer's protocol. Briefly, the cells were cultivated in 96-well plates at 4×10^3 cells/well. Twenty hours after culture, the cells were treated with 50 µmol/L 5-ethynyl-2'-deoxyuridine (EdU; RiboBio, Guangzhou, China) and incubated for 2 h at 37 °C. The cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 for another 20 min. Afterwards, the cells were washed five times with PBS and incubated with 100 µL of 1×Apollo® reaction

cocktail for 30 min at room temperature. Finally, the nuclei of the cells were dyed with 100 µL of Hoechst 33342 (5 µg/mL) for 20 min and visualized with fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

Cell cycle analysis

Forty-eight hours after transfection, the cells were synchronized by serum starvation overnight and induced to re-enter the cell cycle by incubating in medium containing 10% foetal bovine serum for 4 h. Then, the cells were collected, washed with PBS, and fixed in precooled 70% ethanol at 4 °C overnight. The day after, the cells were washed twice with PBS and resuspended in RNase A at 37 °C for 30 min, and then, propidium iodide (PI) was added to the cells in the dark at 4 °C for 30 min. Finally, all samples were analysed by flow cytometry (BD, FACSCanto™ II).

Apoptosis

Apoptosis assays were carried out using the Annexin V-FITC/PI apoptosis detection kit (Nanjing KeyGen Biotech, Inc.) according to the manufacturer's protocol. Briefly, the cells were collected after transfection with siRNA for 48 h, washed twice with PBS, and resuspended in binding buffer. Sequentially, the cells were stained with Annexin V-FITC and PI at room temperature for 15 min and then analysed by flow cytometry (BD, FACSCanto™ II).

Western blot analysis

The cells were lysed in RIPA buffer and centrifuged at 12 000 g for 10 min at 4 °C. The supernatants were collected for Western blotting assays. Equal amounts of proteins were subjected to 10% SDS-PAGE and then transferred to 0.45-µm pore size PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked for 2 h with 5% skim milk in Tris buffered saline containing 0.1% Tween 20 and incubated overnight at 4 °C with anti-mouse GAPDH (Cell Signaling Technology, USA), anti-rabbit LMNB2 (Abcam, USA), and anti-rabbit P21 (Cell Signaling Technology, USA). The membranes were washed 10 min three times with Tris-buffered saline containing 0.1% Tween 20 and incubated for 2h with appropriate secondary antibodies. Specific primary antibodies against LMNB2 (ab151735) and Ki67 (ab16667) were purchased from Abcam. Antibodies against p21 (#2947), p27 (#3686), cyclin D1 (#2978), cyclin E2 (#4132), and Cdk2 (#2546) were obtained from Cell Signaling Technology. Antibodies against GAPDH (60004-1-Ig) were purchased from Proteintech.

RNA extraction and quantitative real-time PCR

TRIzol reagent was used for total RNA extraction (Invitrogen) following the manufacturer's instructions. After RNA purity was measured, a reverse transcription reaction was performed. The PrimeScript™ RT kit (1 µg RNA) and gDNA Eraser (Vazyme) were used. The primers for the p21 promoter fragment were reported(26). The primer sequences are as followed:

5'-TGGAGATCAACGCCTACCG -3' (forward) and

5'-AGCCGCTTCCGCTTACTG -3' (reverse) for LMNB2;

5'-AAGGTCGGAGTCAACGGATTTG-3' (forward) and

5'-CCATGGGTGGAATCATATTGGAA-3' (reverse) for GAPDH.

Real-time PCR was performed using SYBR Green PCR Master Mix with an ABI-7500 qRT-PCR thermal cycler (Vazyme Biotech, Nanjing, China). The relative mRNA level of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control.

Dual-luciferase reporter assays

HCT116 cells were transiently transfected with siRNA specific for LMNB2 and nonspecific control siRNA using siLentFect™ Lipid Reagent for RNAi (Bio-Rad Laboratories, Inc.), p21 promoter plasmid, and Renilla luciferase plasmid. Forty-eight hours post transfection, we collected the cells and measured the activities of both firefly luciferase and Renilla luciferase according to the dual-luciferase reporter assay system (Promega, Madison, WI, USA). The internal standard for transfection efficiency was normalized to Renilla luciferase activity. The p21-luc plasmid (–2400/+11) was a gift from Dr. Baiqu Huang (The Institute of Genetics and Cytology, Northeast Normal University).

Chromatin immunoprecipitation assay

ChIP assays were performed according to the protocol of the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). HCT116 cells cultured in 100 mm dishes (approximately 1×10^7 cells) were crosslinked by adding formaldehyde to a final concentration of 1% and incubated at room temperature for 10 min, washed twice with cold PBS containing protease inhibitors, lysed by ChIP lysis buffer, and sonicated to shear DNA at 4 °C to reduce the average length. Sonicated lysates were then diluted 10-fold with ChIP dilution buffer, and nonspecific binding was reduced with protein A-agarose for 1 h at 4 °C. In this step, 20 µl of lysate was removed as an input control, followed by incubation with anti-LMNB2 or anti-IgG (as a negative control) at 4 °C overnight with rotation. After several washes with a series of buffers, qRT-PCR was performed to amplify the genomic region of p21 flanking the potential LMNB2-binding sites.

In vivo tumour xenograft model

The animal experiments were approved by the Animal Care Committee of Xuzhou Medical University, Xuzhou, China. Female BALB/c nude mice (6–8 weeks old) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd., and maintained under specific pathogen-free conditions. HCT116 cells (5×10^6 cells) with knockdown of LMNB2 and control cells were injected subcutaneously into the flanks of the mice. Tumour volume (V) was monitored every 2 days by measuring the long axis (L) and the short axis (W) with a Vernier caliper and calculated with the following formula: $V = (L \times W^2) / 2$. Twenty days later, the mice were killed, and the tumours were weighed and processed to detect the expression of LMNB2, p21, and Ki67 by IHC.

Statistical analysis

SPSS 23.0 was used for all statistical analyses. The difference between LMNB2 staining of CRC tumours and its corresponding adjacent noncancerous tissues was evaluated by a paired Wilcoxon test. The χ^2 -test was used to evaluate the relationship between the expression of LMNB2 and clinicopathological features. The Kaplan–Meier method and log-rank test were chosen to investigate the correlation between LMNB2 expression and 5-year overall survival and disease-free survival. Univariate and multivariate Cox proportional hazards regression analyses were performed.

Results

LMNB2 expression is significantly increased in colorectal cancer tissues and cell lines

To identify Lamin family members which play important roles in colorectal cancer, TCGA database was used to discover the differently expressed genes in colorectal cancer tissues and normal tissues. All the differently expressed genes in breast cancers were listed in Additional file1: Table S1. GEPIA analysis found that LMNB2 is differentially expressed in colorectal cancer (Figure 1a). To investigate the role of LMNB2 in CRC development, we tested the expression of LMNB2 in 8 pairs of tumour tissues and adjacent tissues and found that LMNB2 expression was significantly increased in the colorectal cancer tissues (Figure 1b). Next, Western blotting analysis showed that compared with that in the normal colorectal epithelial cells (FHC), the expression of LMNB2 in colorectal cancer cell lines (HCT116, DLD1, SW480, SW620, LOVO) was significantly upregulated (Figure 1c). We analysed 226 tumour and adjacent tissues, and immunohistochemical staining showed that LMNB2 was mainly located in the nuclear membrane of the nucleus (Figure 1d). Pairing the tumour tissue with the adjacent tissue and performing a paired Wilcoxon test revealed that the LMNB2 protein expression in the cancer tissue was significantly higher than that in the adjacent tissue ($P < 0.001$) (Figure 1e).

Upregulated LMNB2 expression is positively correlated with clinicopathological characteristics

We used Fisher's exact test to investigate the relationship between LMNB2 expression and clinicopathological parameters in patients with CRC. The clinicopathological characteristics are summarized in Table 1. The expression of LMNB2 is classified into low (immune response score (IRS): 0-6) or high (IRS: 8-12) groups. As shown in Table 1, we found that the low expression rate of LMNB2 protein was 28.3% (64/226), and the high expression rate was 71.7% (162/226). The high expression of LMNB2 protein was positively correlated with tumour diameter ($P < 0.001$, χ^2 -test), TNM stage ($P < 0.001$, χ^2 -test), lymph node metastasis ($P = 0.003$, χ^2 -test), distant metastasis ($P = 0.039$, χ^2 -test) and depth of invasion ($P = 0.015$, χ^2 -test). In contrast, there was no correlation with age, biological sex or tumour differentiation ($P > 0.05$).

LMNB2 functions as an independent prognostic factor for CRC

We used Kaplan-Meier survival analysis and the log-rank test to test whether the expression of LMNB2 is related to the OS and disease-free cumulative survival of patients with colorectal cancer. Our data showed that compared with colorectal cancer patients with reduced LMNB2 protein expression, colorectal

cancer patients with high LMNB2 protein expression had worse OS and disease-free cumulative survival ($P<0.001$) (Figure 1f, g). To further verify whether LMNB2 expression is an independent prognostic factor of colorectal cancer, we used univariate and multivariate Cox regression models to confirm the prognostic value of LMNB2 expression in colorectal cancer. Univariate Cox regression analysis showed that LMNB2 expression, TNM stage, lymph node metastasis, tumour diameter, distant metastasis and depth of invasion are important prognostic factors affecting OS and DFS in patients with colorectal cancer (Table 2). In the multivariate Cox regression model, our data further confirmed that after adjusting for TNM stage, lymph node metastasis, tumour diameter, distant metastasis and depth of invasion, the expression of LMNB2 was still associated with the OS of patients with colorectal cancer ($P=0.003$, hazard ratio (HR)=0.394, 95% confidence interval (CI)=0.211-0.735) and DFS ($P=0.002$, HR=0.417, 95% CI=0.238-0.731) as independent and meaningful prognostic biomarkers (Table 3). In summary, our results confirmed that LMNB2 expression may be a potential independent prognostic factor for OS and DFS in colorectal cancer patients.

LMNB2 promotes the proliferation of colorectal cancer cells in vitro

Since the expression of LMNB2 is related to tumour diameter, we explored the role of LMNB2 in the proliferation of colorectal cancer cells. HCT116 and DLD1 cells were transiently transfected with small interfering RNAs (siRNAs) targeting LMNB2 (siLMNB2#1, siLMNB2#2) or control siRNA (siCtrl). The results revealed that LMNB2 expression was significantly reduced in the cells transfected with LMNB2 siRNA compared with the control cells (Figure 2a, b). In CCK-8 proliferation assays, the proliferation of HCT116 and DLD1 cells decreased significantly after knocking down LMNB2 expression (Figure 2c). The EdU incorporation assay showed that compared with the corresponding control cells, the EdU-positive HCT116 and DLD1 cells in the LMNB2 downregulation group were significantly reduced (Figure 2d, e). The clone formation assay further verified the effect of LMNB2 on the proliferation of colorectal cancer cells. The results showed that the colony forming ability of the HCT116 cells transfected with LMNB2 shRNA was weaker than that of the control group; similar results were also observed in DLD1 cells (Figure 2f). These results indicated that LMNB2 plays a vital role in the proliferation of colorectal cancer cells.

LMNB2 regulates the expression of P21 in colorectal cancer cells

To identify potential mRNA targets regulated by LMNB2, we performed RNA sequencing of DLD1 cells. Three knockdown cell lines and three control stable cell lines were selected for RNA sequencing. A total of 7445 genes were identified from mRNA sequencing. Among them, 3579 genes were upregulated and 3866 genes were downregulated (Figure 3a). All genes are listed in Additional file 2: Table S2. Some DEGs between the control group and the LMNB2-knockdown group are shown. All DEGs were subjected to functional enrichment analyses via DAVID. For a deeper understanding of these DEGs, we conducted a Panther pathway enrichment analysis. These genes are differentially enriched in certain cancer-related pathways. For example, cell cycle pathways showed a strong correlation (Figure 3b). Among these genes, P21 (CDKN1A) was significantly upregulated after LMNB2 knockdown (Figure 3c). In the TCGA database, the expression of p21 in the tissues of colorectal cancer patients was less than that in the normal

colorectal tissues (Figure 3d). The GEPIA database shows that LMNB2 is negatively correlated with p21 (Figure 3e). Since GSEA analysis showed that LMNB2 amplification was positively correlated with genes related to "KEGG cell cycle" and "GO cell cycle G1-S transition", we further studied the effect of LMNB2 on CRC cell cycle regulation (Figure 3f, g).

LMNB2 regulates the p21-mediated cell cycle and promotes cell proliferation

Flow cytometry showed that the downregulation of LMNB2 expression resulted in G1 arrest, indicating that LMNB2 is involved in cell cycle regulation. Taken together, these results indicated that inhibiting LMNB2 inhibits the proliferation of CRC cells by arresting the colorectal cancer cell cycle in the G1 phase (Figure 4a). However, since apoptosis also has an important effect on cell proliferation, we used flow cytometry to analyse the effect of LMNB2 on cell apoptosis. The results showed that there was no significant change in apoptosis after silencing LMNB2 (Figure 4b). In addition, Western blot analysis showed that knockdown of LMNB2 in the HCT116 and DLD1 cell lines significantly increased the expression of P21. Furthermore, the downregulation of LMNB2 resulted in significant accumulation of p27 protein, while it significantly reduced the expression of cyclin D1, cyclin E2, and cyclin-dependent kinase 2 (CDK2). Real-time quantitative PCR confirmed these results, and the results showed that LMNB2 negatively regulates p21 and p27 at the mRNA level. In addition, the expression of apoptotic factors, including cleaved caspase 3, cleaved caspase 7, cleaved caspase 9 and bcl-2, did not change significantly (Figure 5a, b).

LMNB2 transcriptionally inhibits p21 in CRC cells in vitro

Cell cycle progression is regulated by the activation of multiple CDK/cyclin complexes(27). To understand the molecular mechanism of LMNB2's effects on the cell cycle in G0/G1 phase, we focused on the universal cell cycle inhibitor p21, which is a tumour suppressor involved in regulating G0/G1 phase arrest and cell proliferation(28). Previous studies have shown that p21 is an over-regulated gene, and its gene promoter is affected in many ways. Based on this, we investigated whether LMNB2 participates in the cell cycle process by regulating the p21 promoter. We analysed the promoter region of the human p21 gene and cloned it into the luciferase reporter plasmid (Figure 5c). Subsequently, the dual-luciferase reporter experiment showed that transfection of LMNB2 targeting siRNA significantly increased the activity of the p21 promoter (Figure 5d), indicating that LMNB2 plays a key role in regulating the expression of p21 in colorectal cancer at the transcriptional level. We analysed the promoter region of the human p21 gene and designed a potential binding region. These regions are denoted as p1, p2, p3, p4, p5, and p6. To determine whether LMNB2 can be recruited to the potential binding region of the p21 promoter, we performed chromatin immunoprecipitation (ChIP) analysis in HCT116 LMNB2 knockout cells and control cells. The ChIP quantitative PCR results showed that LMNB2 mainly binds to the p3- and p6-binding regions of the p21 promoter (Figure 5e).

LMNB2 promotes the growth of colorectal cancer in vivo and affects the proliferation of colorectal cancer cells

We established a stable cell line in which LMNB2 was knocked down in HCT116 cells in preparation for further research. Western blot analysis showed that LMNB2 was knocked down in a stable cell line (Figure 6a). We subcutaneously injected the stable LMNB2-KD cells and the control cells mixed with Matrigel into BALB/c nude mice. After 6 days, we measured the maximum length and width of the subcutaneous tumour every other day. The mice were sacrificed 20 days later, and the tumours formed by stable LMNB2 knockout cells were significantly smaller and lighter than those of the control group (Figure 6b). The tumour growth and tumour volume of the HCT116 cell group injected with the stable LMNB2 gene were significantly lower than those of the control group. (Figure 6c, e). We randomly selected 4 pairs of tissues for Western blot experiments and found that LMNB2 expression was low in the experimental group (Figure 6d). In addition, the shLMNB2 and shCtrl tumour tissue sections were subjected to immunohistochemical staining to detect the expression of LMNB2, p21 and the nuclear proliferation marker Ki67. Representative images showed that knockout of LMNB2 resulted in decreased staining intensity of LMNB2 and Ki67 in the resected tumour and increased staining intensity of p21 compared with those of the control group (Figure 6f). In summary, these results indicated that knockout of the LMNB2 gene inhibits the proliferation of colorectal cancer cells in vivo.

Downregulation of P21 reverses the inhibition of proliferation by LMNB2 gene knockdown

To evaluate the effect of p21 in suppressing the proliferation induced by LMNB2 silencing, we silenced p21 in HCT116 and DLD1 cells with silenced LMNB2 by siRNA (Figure 7a). The results showed that transiently transfected p21 siRNA can reverse the inhibited proliferation due to LMNB2 gene knockout in HCT116 and DLD1 stable cell lines (Figure 7b, c, d). These results indicated that p21 plays an important role in the proliferation of colorectal cancer cells regulated by LMNB2.

Discussion

The occurrence and development of tumours is a multifactor and multistep complex process that includes oncogene activation and tumour suppressor gene inactivation. Therefore, exploring the exact molecular mechanism of colorectal cancer progression is important for discovering promising prognostic biomarkers and potential new therapeutic targets for this disease.

Lamin is overexpressed in most cancers and has the ability to maintain cancer cell homeostasis. In particular, laminin maintains cell differentiation, proliferation and movement in tumours, which is essential for aggressive tumors(12).Lamin B tethering may differentially affect gene expression by forming complexes with different chromatin binding proteins, epigenetic regulators and transcription factors in specific chromatin regions(29). LMNB2 is involved in chromatin remodelling and the rupture and reorganization of the nuclear membrane during mitosis, which is necessary for eukaryotic cell proliferation. LMNB2 has been shown to play an important role in the occurrence and development of tumours. In non-small cell lung cancer, overexpression of LMNB2 promotes cell proliferation and migration and invasion(24, 25). However, there is no research on the relationship between LMNB2 expression and the progression of colorectal cancer.

In this study, the expression of LMNB2 protein in colorectal cancer tissues was higher than that in adjacent tissues, and the high expression of LMNB2 was related to tumour size, TNM stage and lymph node metastasis. Both TCGA database analysis and patient samples collected in our hospital showed that the LMNB2 gene was upregulated in colorectal cancer. In addition, univariate and multivariate Cox regression analysis showed that high expression of LMNB2 is an independent biomarker of poor prognosis for patients with colorectal cancer. These findings indicate that LMNB2 plays an important role in the development of colorectal cancer and can be used as a potential clinical prognostic indicator for patients with colorectal cancer undergoing radical resection. The progress of colorectal cancer accompanies or promotes the infinite proliferation and metastasis of cancer cells(30). Based on our findings in the clinical data and the consensus that the progression rate of colorectal cancer depends on the growth of cancer cells, we conducted a series of cell line and animal experiments to explore the possible role of LMNB2 in colorectal cancer. Our current data showed that in cultured CRC cells, LMNB2 blockade downregulates the proliferation and colony formation of CRC cells. In addition, the knockout of LMNB2 significantly reduced the growth of colorectal cancer in vivo. These findings indicate that LMNB2 may promote the progression of colorectal cancer by promoting cell proliferation.

Cell cycle disorders can lead to abnormal cell proliferation, and abnormal cell proliferation is closely related to the occurrence of tumours. Any interference with the programs involved in cell cycle regulation may lead to abnormal cell proliferation(31). P21, also known as cyclin-dependent kinase inhibitor 1 (CDKN1A), is an important regulator of the G1/S transition phase of the cell cycle, a member of the cyclin/cyclin-dependent kinase inhibitor (CKI) family and considered an important regulator of cell growth inhibition(32, 33). It has been confirmed that increased expression of p21 can inhibit cell proliferation and growth. In our study, we examined the role of LMNB2 in colorectal cancer and found that LMNB2 can be recruited to the P21 promoter region to regulate the expression of P21 at the transcriptional level. To clarify the mechanism of LMNB2 in the proliferation of colorectal cancer, we performed mRNA sequencing using LMNB2 knockout cells and control HCT116 cells. RNA sequencing and subsequent pathway analysis were used to determine the main targets regulated by LMNB2. In addition, LMNB2 is a favourable factor for patients with colorectal cancer. It has been confirmed that LMNB2 can promote the proliferation of colorectal cancer. LMNB2 gene knockout inhibits the proliferation of colorectal cancer cells in vivo and in vitro. In summary, our study has identified members of the Lamin family that play an important role in the development and carcinogenesis of colorectal cancer. As a member of the Lamin family, LMNB2 plays a role in promoting colorectal cancer. Our research emphasizes the role of LMNB2 in promoting tumour proliferation. RNA sequencing and subsequent pathway analysis were used to determine the main targets regulated by LMNB2. P21, as the direct target of LMNB2, reveals the main mechanism by which LMNB2 regulates target genes. Elevated LMNB2 can be directly recruited to the p21 promoter to inhibit its transcription in CRC cells. Understanding the role of LMNB2 in colorectal cancer cell proliferation will help develop new colorectal cancer treatments.

In conclusion, our study shows for the first time that the abnormal expression of LMNB2 promotes the proliferation and development of colorectal cancer. LMNB2 promotes cell proliferation of colorectal

cancer by inducing p21-mediated cell cycle progression. These findings reveal the promise of LMNB2 as a potential biomarker and target for the prognosis and treatment of colorectal cancer.

Abbreviations

CCK-8: Cell Counting Kit-8; DMEM: Dulbecco Modified Eagle Medium; RPMI: Roswell Park Memorial Institute; PBS: phosphate buffer saline; RIPA: RIPA Lysis Buffer; SDS-PAGE: sodium dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis; PVDF: Polyvinylidene fluoride; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; SPSS: Solutions Statistical Package for the Social Sciences; GEPIA: Gene Expression Profiling Interaction Analysis; OS: Overall survival; DFS: Disease-free survival; TCGA: The Cancer Genome Atlas; GSEA: Gene Set Enrichment Analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: gene ontology.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Further details are available from the corresponding author upon request.

Authors' contributions

CHD, TJ, HY provided study concept and design. CHD, TJ and HY collected and analysed the data. CHD, TJ, HY and HS interpreted the data. CHD, TJ, HY, HS, and YZ performed the experiments. PCS, YXX and HG collected the patients' samples. CHD, LYL and LZ wrote the paper. All authors approved the final version of paper.

Ethics approval and consent to participate

This study was conducted in compliance with the declaration of Helsinki. Informed consent was obtained from all subjects. The ethics approval statements for human subjects were provided by the Ethnic Committee of the Affiliated Hospital of Xuzhou Medical University. The ethics approval statements for animal work were provided by the Institutional Animal Care and Use Committee of Xuzhou Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1

Variables	cases	LMNB2 expression(n = 226 cases)		p ^a
		Low(%)	High(%)	
All patients	226	64(100)	162(100)	
Age(years)				0.285
≤ 60	101(45)	25(39)	76(47)	
>60	125(55)	39(61)	86(53)	
Gender				0.332
Males	114(55)	29(45)	85(52)	
Females	112(54)	35(55)	77(48)	
Tumor diameter				< 0.001
≤ 5 cm	128(57)	48(75)	80(49)	
> 5 cm	98(43)	16(25)	82(51)	
Lymph node metastasis				0.003
N0	139(62)	49(90)	90(49)	
N1/N2/N3	87(38)	15(10)	72(51)	
TNM stage				< 0.001
I~II	120(53)	48(76)	72(44)	
III~IV	106(47)	16(24)	90(56)	
Differentiation ^b				0.198
Poor	32(14)	12(19)	20(12)	
Moderate/high	189(86)	50(81)	139(88)	
Distant metastasis				0.039
M0	207(84)	63(98)	144(89)	
M1	19(16)	1(2)	18(11)	
Depth of invasion				0.015

^a Two-sided Fisher's exact tests

^bThe type of differentiation of cancer in five patients cannot be assessed

Variables	cases	LMNB2 expression(n = 226 cases)		p ^a
		Low(%)	High(%)	
T1/T2	56(25)	23(36)	33(20)	
T3/T4	170(75)	41(64)	129(80)	
^a Two-sided Fisher's exact tests				
^b The type of differentiation of cancer in five patients cannot be assessed				

Table 2

Variables ^a	Overall survival		Disease-free survival	
	HR(95% CI)	P	HR(95% CI)	P
LMNB2	2.834(1.572–5.109)	0.001	2.376(1.408,4.009)	0.001
Age	1.522(0.934–2.479)	0.092	1.787(1.120,2.853)	0.015
Gender	0.969(0.608–1.545)	0.896	1.065(0.683–1.661)	0.782
LNM	1.994(1.208–3.291)	0.007	1.329(0.834,2.116)	0.231
Distant metastasis	1.779(0.871–3.634)	0.114	1.687(0.866–3.283)	0.124
TNM stage	2.8(1.712–4.610)	0.033	1.635(1.042,2.564)	0.032
Differentiate	1.09(0.556–2.137)	0.803	0.65(0.353,1.195)	0.165
Tumor Diameter	2.426(1.418–3.937)	< 0.001	1.294(0.823,2.033)	0.264
Depth of invasion	1.783(1.078–2.949)	0.024	1.893(1.177,3.044)	0.009
HR hazard ratio, CI confidence interval, LNM lymph node metastasis				
^a LMNB2: low vs high; age: ≤60 vs > 60; gender: male vs female; LNM: N0 vs N1, N2, N3; depth of invasion: T1–T2 vs T3–T4; distant metastasis: M0 vs M1; differentiate: poor vs moderate and high; TNM stage was ranked as I–II vs III–IV; tumor diameter: ≤5 v s > 5				

Table 3

Variables ^a	Overall survival		Disease-free survival	
	HR(95% CI)	P	HR(95% CI)	P
LMNB2	0.394(0.211–0.735)	0.003	0.417(0.238–0.731)	0.002
Age	1.249(0.759–2.056)	0.132	2.142(1.303–3.523)	0.003
LNM	1.78(0.933–2.670)	0.089	1.127(0.661–1.922)	0.659
TNM stage	1.709(0.997–2.932)	0.051	1.555(0.915–2.64)	0.102
Distant metastasis	1.158(0.556–2.414)	0.695	1.393(0.699–2.776)	0.347
Tumor Diameter	1.695(0.986–2.913)	0.056	0.871(0.522–1.455)	0.598
Depth of invasion	1.467(0.870–2.476)	0.151	1.643(1.005–2.686)	0.048
HR hazard ratio, CI confidence interval, LNM lymph node metastasis				
^a LMNB2: low vs high; age: ≤60 vs >60; LNM: N0 vs N1, N2, N3; depth of invasion: T1–T2 vs T3–T4; differentiate: poor vs moderate and high; TNM stage was ranked as I–II vs III–IV; tumor diameter: ≤5 v s > 5				

Figures

Figure 1

Expression of LMNB2 is upregulated in CRC and negatively associated with overall and disease-free survival in CRC. a. The GEPIA database shows that LMNB2 is highly expressed in colorectal cancer tissues. b. Detection of LMNB2 protein levels in 8 cancer tissues and paired normal colon tissues by western blot. c. The expression level of LMNB2 was detected by western blot in five CRC cell lines and a normal colon epithelial cell line (FHC). d. LMNB2 immunohistochemical staining shows TMA. Note: the

upper screen, the magnification is $\times 100$; the lower screen, the magnification is $\times 400$. e. Distribution of LMNB2 staining intensity difference between colorectal cancer tissue and paired adjacent tissues. Note: N is the paired adjacent non-cancerous tissue. The expression level of LMNB2 in colorectal cancer tissues was significantly higher than that in the corresponding adjacent tissues (paired Wilcoxon test, $P < 0.001$). f. High expression of LMNB2 is associated with poorer overall cumulative survival of patients with colorectal cancer ($P < 0.001$, log-rank test). g. High expression of LMNB2 is associated with poor disease-free cumulative survival in patients with colorectal cancer ($P < 0.001$, log-rank test)

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Figure 2

LMNB2 promotes the proliferation of colorectal cancer cells. a. Western blotting confirmed that the protein level of LMNB2 in HCT116 and DLD1 cells was downregulated. b. Real-time quantitative PCR confirmed that the mRNA levels of LMNB2 in HCT116 and DLD1 cells were knocked out. c-f. gene knockout can significantly inhibit the proliferation of HCT116 and DLD1 cells. All experiments are in triplicate. The data are shown as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$

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Figure 3

Differentially expressed genes and pathways regulated by LMNB2. a. mRNA sequencing identified 7445 differentially expressed genes. Among them, 3579 genes (red) were up-regulated and 3866 genes (blue) were down-regulated. b. Kyoto Encyclopedia of Genes and Genomes pathway analysis was performed for screening DEGs using the PANTHER database. c. Heat map of differentially expressed genes after LMNB2 gene knockout. CDKN1A and RBMS2 expression heat map of MRNA sequencing. d. GEPIA

database shows that P21 is low in colorectal cancer tissues. e. GEPIA database shows that LMNB2 and P21 are negatively correlated. f, g. GSEA analysis showed that the LPCAT1 amplification status was positively correlated with genes related to “KEGG CELL CYCLE” and “GO CELL CYCLE G1-S TRANSITION”

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Figure 4

LMNB2 can accelerate the cycle of colorectal cancer cells, but has no effect on cell apoptosis. a. Flow cytometry analysis after Annexin FITC and PI staining found that after LMNB2 gene knockout, the number of cells in G0/G1 phase increased, while the number of cells in S phase and G2 phase decreased. b. Flow cytometry analysis and Annexin PI staining results showed that LMNB2 gene knockout had no significant effect on the apoptosis of colorectal cancer cells. All experiments were performed independently three times. The data are the mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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Figure 5

LMNB2 gene knockout can induce cell cycle arrest in G0/G1 phase and inhibits the transcription of p21. a. Western blotting was used to detect the expression of cell cycle and apoptosis-related genes. GAPDH was used as a reference control. b. Real-time fluorescence quantitative PCR detects the relative expression of cell cycle-related genes at the mRNA level. GAPDH was used as a reference control. c. The complete sequence of the human p21 promoter. P1-6 shows the p21 promoter region detected by two pairs of primers. d. The activity of P21 promoter (LMNB2 2400/11) in -116 cells was enhanced after LMNB2 gene knockout. e. Three-dimensional chip quantitative PCR analysis of the binding of LMNB2 at p1, p2, p3, p4, p5 and p6. Western blot detection of LMNB2 in chip detection. The mean $\pm$ standard deviation of three experiments is given. *P<0.05, **P<0.01, ***P<0.001

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Figure 6

Knockout of LMNB2 gene can inhibit tumor formation in colorectal cancer cells. a. Western blotting confirmed that the LMNB2 gene was knocked out in the HCT116 stable cell line at the protein level. b. Gross observation of subcutaneous tumors of nude mice on HCT116 cells transfected with shLMNB2 and shCtrl lentivirus (10 mice per group). The mice were sacrificed 20 days after injection, and the transplanted tumors were collected. c. Analyze the weight of the transplanted tumor. d. Detection of LMNB2 protein levels in 4 cancer tissues and paired normal colon tissues by western blot. e. The volume of transplanted tumor in nude mice. The calculation formula of transplanted tumor volume is: $V = (L \times W^2) / 2$. f. Tumor sections were immunohistochemically stained with antibodies against LMNB2, p21 and Ki67, and a representative image was displayed (magnification: 400 times). * $P < 0.05$. L represents the long axis, W represents the short axis.

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Figure 7

Down-regulation of P21 can reverse the inhibition of proliferation caused by LMNB2 gene knockout. a. Western blotting confirmed the presence of LMNB2 and p21 gene knockout in HCT116 and DLD1 stable cell lines at the protein level. b-d. transiently transfected with p21siRNA can reverse the inhibitory effect of LMNB2 gene knockout on the proliferation of HCT116 and DLD1 cells. The data is shown as the mean $\pm$ standard deviation from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001

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