

Molecular mechanism of LKB1 in the invasion and metastasis of colorectal cancer

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Abstract. The occurrence of colorectal cancer (CRC) is associated with a variety of oncogenes and tumor-suppressor genes. As a tumor-suppressor gene, the liver kinase B1 gene (LKB1, also known as serine/threonine kinase 11, STK11) is closely related to tumor angiogenesis, invasion and metastasis, but its molecular mechanisms remain unclear. The aim of the present study was to investigate the effects of LKB1 on the invasion and metastasis of CRC, and to explore its molecular mechanisms. By detecting the expression of LKB1 in CRC, we can provide a reference index for diagnosing the depth of invasion and lymph node metastasis. Immunohistochemistry results indicated that LKB1 expression was strongly positive in normal colon tissue and that it inhibited the production of CRC. Immunocytochemical staining showed that the expression of LKB1 was significantly decreased in adenocarcinoma and mucinous adenocarcinoma tissues, and this reduced expression induced the invasion and metastasis of CRC. In the present study, LKB1 small interfering RNA (LKB1 siRNA) was transfected into LoVo cells to observe the effect of LKB1 on the invasion and metastasis of CRC. LKB1 silencing decreased the phosphorylation of AMP-activated protein kinase (p-AMPK) in its downstream pathway, which increased the phosphorylation of protein kinase B (p-AKT) and promoted tumor cell proliferation, enhancing the migration and invasion of CRC. The present study also explored the role of metformin in the LKB1 signaling pathway. Metformin inhibits the invasion and metastasis of CRC by activating p-AMPK, thereby inhibiting the activation of p-AKT. These results suggest that LKB1 plays an important role in the invasion and metastasis of CRC by activating AMPK, negatively regulating the AKT

signaling pathway and regulating gene expression. Mutation or deletion of LKB1 is expected to be a novel therapeutic target or clinical biomarker for the prevention of the invasion and metastasis of CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in humans. When diagnosed with advanced metastatic disease, CRC patients traditionally have a poor prognosis, with 5-year survival rates in the range of 5 to 8% (1,2). Clinical studies have shown that the main cause of death in patients with CRC is tumor invasion and metastasis to other tissues and organs (3). In addition to surgical resection of the primary tumor, the clinical key to improving patient survival and curing CRC is to inhibit angiogenesis, cutting off the transfer route and looking for new targets for effective drug treatment (4). The occurrence of CRC involves a variety of oncogenes and tumor-suppressor genes, although the underlying molecular mechanisms remain unclear (5,6). The study of targeted gene therapy can provide a theoretical basis for the treatment of the invasion and metastasis of CRC.

As a tumor-suppressor gene, the liver kinase B1 gene (LKB1, also known as serine/threonine kinase 11, STK11) is closely related to tumor angiogenesis, invasion and metastasis (7,8). The loss of LKB1 gene expression is related not only to the occurrence of a variety of tumors, such as CRC and thyroid carcinoma, but also to tumor angiogenesis and metastasis (9-11). Knockdown of LKB1 was found to enhance cell migration in human colon cancer HCT116 cells (12). The molecular mechanism by which the LKB1 pathway acts on angiogenesis and metastasis in CRC remains unclear. It is evident that small interfering RNAs (siRNAs) play an important role in cancer initiation and progression via the regulation of tumor-suppressor genes or oncogenes (13-15). In the present study, the LKB1 gene was silenced by siRNA, and the mechanism by which LKB1 affects the downstream invasion and metastasis of CRC cells was observed.

LKB1 has recently been identified as an upstream activating protein kinase of AMP-activated protein kinase (AMPK) (16). The study found that AMPK and the development of CRC are closely related (17,18). Our studies revealed an important role played by AMPK alpha 1 (AMPK α 1) in cell biology, connecting

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two hallmarks of tumor cells, namely, hyperproliferation and DNA damage, which may be due to reduction in the amount of p21 (19). These findings have important implications for understanding the molecular mechanisms by which AMPK acts as a promising tumor suppressor or senescence blocker. Su *et al* found that AMPK was closely related to angiogenesis in endothelial cells (20). LKB1 is a key regulator of the AMPK signaling pathway in some tissues, including liver, skeletal muscle, myocardium and various cancerous tissues (21,22). Our data indicated that protein kinase B (AKT) is involved in the angiogenesis and metastasis of colon cancer. Aberrant AKT activation was found to promote the growth of a variety of LKB1-deficient tumors (23) by promoting the activation of vascular endothelial growth factor and angiogenic factors (24). Therefore, it is important to study the expression and regulation of LKB1 to clarify the mechanisms underlying the invasion and metastasis of CRC.

In the present study, immunohistochemistry (IHC) was used to detect the expression of the tumor-suppressor gene LKB1 in different colon tissues. CRC LoVo cells were transfected with LKB1 small interfering RNA (LKB1 siRNA). Metformin has the potential mechanism to inhibit tumor cell growth and reduce protein synthesis in CRC. The present study used LoVo cells treated with metformin to study the expression and biological significance of LKB1-AMPK-AKT to clarify the metastatic mechanism of CRC. We speculated that LKB1 activates AMPK, acting as a negative regulator of the AKT pathways and affecting the invasion and metastasis of colon cancer cells. In the present study, we explored the possibility of the prevention and treatment of the invasion and metastasis of colon cancer via the LKB1 pathway.

Materials and methods

Materials. The colon cancer tissue microarray was purchased from Fanpu Biotech, Inc. (cat. no. COC961; Guilin, China). The SP IHC kit and primary antibody dilution buffer were purchased from Sangon Biotech (Shanghai, China). The LoVo human CRC cell line was obtained from CHI Scientific (cat. no. CL-0144, Wuxi, China). The artificially synthesized LKB1 siRNA and RNase-free water were obtained from RiboBio (Guangzhou, China). Lipofectamine™ 2000 was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All primary antibodies [LKB1 (dilution 1:1,000; cat. no. 3050S), p-LKB1 (dilution 1:1,000; cat. no. 3482S), AMPKa (dilution 1:1,000; cat. no. 2532S), p-AMPKa (Thr172, dilution 1:1,000; cat. no. 2535S), AKT (dilution 1:1,000; cat. no. 9272S), p-AKT (dilution 1:2,000; cat. no. 4060S)] were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Prestained Dual Color Protein Molecular Weight Marker was purchased from Thermo Fisher Scientific, Inc. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories, Co., Ltd. (Kyushu, Japan). Metformin was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The study was approved by the Ethics Committee of Hubei University of Science and Technology (Xianning, China).

IHC. The rabbit anti-human LKB1 monoclonal antibody (dilution 1:200; cat. no. ab185734) was purchased from Sangon Biotech. The colorectal carcinoma chip was purchased

from Fanpu Biotech, Inc. This chip included samples from 65 patients with different types of colorectal tumors and samples of normal and inflammatory polyps from 31 patients. The samples were adenocarcinoma and mucinous adenocarcinoma tissues from 65 patients. Of the 65 patients, 35 were male and 30 were female; 36 were younger than 60 years, while 29 were older than 60 years. The TNM staging of colon cancer was carried out according to the 8th edition of the AJCC Cancer Staging Manual (25). In the present study, we observed the effect of LKB1 on metastasis of CRC. Therefore, the lymph node metastasis status in the present study was divided into two types: No metastasis and metastasis, and no detailed distinction was made. The 65 colorectal tumor samples were composed of 20 cases of adenocarcinoma with pathological grade I, 31 cases of adenocarcinoma with pathological grade II, and 14 cases of adenocarcinoma with pathological grade III. The tissues were cut into 4- μ m-thick slices. The IHC SP method was performed according to the manufacturer's instructions, with known positive tissue sections as a positive control and phosphate-buffered saline (PBS) instead of primary antibody as a negative control. The procedure was conducted as follows: i) Dewaxing and hydration with a graded ethanol series moving from a high ethanol concentration to a low ethanol concentration; ii) antigen repair; iii) incubation with normal goat serum solution at 37°C for 10 min; iv) application of the primary antibody LKB1; dilution 1:200) at 4°C and incubation overnight, washing with PBS 3 times for 3 min each time, incubation with the mouse anti-rabbit secondary antibody (dilution 1:200; cat. no. BM2004; Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) at 37°C for 1 h and washing with PBS 3 times for 3 min each time; v) application of the horseradish peroxidase-labeled streptomycin-avidin working solution and washing with PBS 3 times for 3 min each time; vi) DAB/H₂O₂ reactive dyeing followed by thorough rinsing with water, hematoxylin staining, dehydration, transparent and drying; vii) mounting with a neutral gum with no bubbles. The sections were scanned at magnification (x200) using an light microscopy (Olympus Corp. Tokyo, Japan). The intensity of the staining as well as the percentage of positive cells was recorded. Staining intensity was scored from 0 to 3+; the intensity score was established as follows: 0 if tumor cells had complete absence of staining or faint staining intensity in <10%; 1+ if >10% of tumor cells had faint staining; 2+ if tumor cells had moderate staining; and 3+ if tumor cells had strong staining. Tumors with 1+, 2+ and 3+ expression were interpreted as positive for LKB1 antibody expression, and tumors with no expression (0 score) were interpreted as negative.

Cell culture. LoVo cells were maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS; CHI Scientific) with 1% penicillin-streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA). The cells were grown in monolayer cultures with 5% CO₂ in a humidified 37°C incubator. Every 2-3 days, the cells were subcultured. When the cells reached the logarithmic growth phase, 0.25% trypsin (Gibco Invitrogen; Thermo Fisher Scientific, Inc.) was applied for 1-3 min. The cells were resuspended in RPMI-1640 containing 10% FBS at a cell concentration of 1x10⁴ cells/ml.

The experimental groups were as follows: i) blank control group, untransfected LoVo cells; ii) metformin group, LoVo cells treated with 20 mmol/l metformin; iii) LKB1 siRNA group, LoVo cells transfected with LKB1 siRNA; and iv) LKB1 siRNA+metformin group, LoVo cells transfected with LKB1 siRNA were also treated with 20 mmol/l metformin.

LKB1 siRNA synthesis and transfection. The cells were divided into the following 3 groups: i) blank control group, untransfected LoVo cells; ii) negative control group, LoVo cells transfected with Lipofectamine™ 2000; iii) positive control group (LKB1 siRNA group), transfected with LKB1 siRNA. Appropriate positive and negative controls were run simultaneously. In total, 3 groups of cells were transfected with siRNAs the sequences of which were specific for LKB1 (namely, siRNA-001, siRNA-002 and siRNA-003). Another group of cells was transfected with a fluorescein amidite (FAM)-labeled non-specific siRNA that served as the negative control (NC) siRNA. The sequences of the siRNAs used in the present study were as follows: siRNA-001 sense, 5'-GAAGAAGGAAAUUCAACU A-3' and antisense, 5'-UAGUUGAAUUCCUUCUUC-3'; siRNA-002 sense, 5'-GCUGGUUCCGGAAGAAACA-3' and antisense, 5'-UGUUUCUCCGGAACCAGC-3'; siRNA-003 sense, 5'-GGACUGACGUGUAGAACAA-3' and antisense, 5'-UUGUUCUACACGUCAGUCC-3'. In the present study, the risk of this potential off-target effect of siRNA affected the application of siRNA in this technology gene therapy field. LKB1 siRNA was synthesized by Guangzhou RiboBio Co., Ltd. The company is careful to avoid the off-target effect when designing siRNA, and the company offers siRNA modification. The company conducts product quality testing after the product is produced. For cell transfection, the cells were plated on 6-well plates (2×10^5 cells for LoVo) in RPMI-1640 with 10% FBS. After 12 h, 10 μ l of siRNA/well was incubated with 100 μ l of Opti-MEM, and 5 μ l of Lipofectamine™ 2000 was diluted with 100 μ l of Opti-MEM; 200 μ l of the above mixture was added/well according to the manufacturer's instructions. The transfected cells were incubated in a humidified 37°C incubator with 5% CO₂ for 48 h.

Examination of morphological changes. Following the application of LoVo-Con siRNA or LKB1 siRNA for 48 h, an inverted phase-contrast microscope (Nikon Corporation, Tokyo, Japan) was utilized to observe the morphological changes in the cells. Images were captured using a digital camera (Nikon Corporation) at a magnification of x200.

CCK-8 assay. The effect on cell proliferation and toxicity due to metformin were assessed using a CCK-8 assay. Briefly, 3×10^4 cells/well were seeded in 96-well culture plates. After overnight incubation, the cells were treated with varying concentrations of metformin for 24, 48 and 72 h. The concentrations of metformin were 0, 5, 10, 15, 20 and 25 mmol/l. A total of 20 μ l of CCK-8 working solution was added to each well, followed by incubation for 4 h at 37°C, and the absorbance was finally measured at 450 nm using a Model 3550 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Migration and invasion assays. Transwell chambers (Corning, Inc., Corning, NY, USA) were used to perform the cell migration

and invasion assays. The cells were inoculated into 6-well plate with RPMI-1640 containing 10% FBS at a cell concentration of 2×10^4 cells/ml. First, 800 μ l of RPMI-1640 medium plus 10% FBS was added to a 24-well plate, and then the plate was inserted into the Transwell chamber. After 1 h, 200 μ l of cell suspension was added to the upper Transwell chamber. The cells were grown in monolayer cultures with 5% CO₂ in a humidified 37°C incubator. After 24 h, the Transwell chamber was washed once with sterile PBS. The cells that had not migrated were wiped off the upper surface with a clean cotton ball, and then a 10% methanol solution was used to fix the migrated cells for 30 min. The film was carefully cut and a drop of 5% crystal violet dye was added to the membrane. The cells were incubated at room temperature for 20 min, and then washed once with PBS. The slide was fixed with neutral gum. Finally, the purple-blue cells were observed and counted under the multifield view of the microscope. The number of tumor cells was calculated in 5 random fields at a magnification of x200, using an inverted microscope (Olympus DP7; Olympus Corp.) and expressed as the average number of cells/field of view. The number of migrating cells present in 9 random fields was counted, and the experiment was repeated ≥ 3 times. Invasion assays were performed using the same protocol, except with Matrigel (BD Biosciences, San Jose, CA, USA)-coated Transwell chambers and incubation for 24 h at 37°C.

Western blot analysis. Protein lysates from cells were extracted in ice-cold lysis buffer [10 μ l PMSF, 10 μ l NaVO₃, 10 μ l phosphatase inhibitor cocktail and 10 μ l protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) in 1 ml RIPA]. Total protein was subjected to SDS-PAGE followed by immunoblotting. The total protein content was quantitated according to the Bradford method. Purified protein lysates (40 μ g) were separated electrophoretically on 12% denaturing SDS polyacrylamide gels (separating gel) and 5% denaturing SDS polyacrylamide gel (concentrated gum). Then, the protein was transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk, the membranes were probed with primary antibodies [LKB1 (dilution 1:1,000); p-LKB1 (dilution 1:1,000); AMPKa (dilution 1:1,000); p-AMPKa (Thr172, dilution 1:1,000); AKT (dilution 1:1,000); p-AKT (dilution 1:2,000); β -actin mouse monoclonal antibody (dilution 1:2,000; cat. no. BM0627; Wuhan Boster Biological Technology, Co., Ltd.)] at 4°C and incubation overnight. The immunoblotted proteins were visualized using the goat anti-mouse secondary antibody (dilution 1:5,000; cat. no. BA1001; Wuhan Boster Biological Technology, Co., Ltd.) or mouse anti-rabbit secondary antibody (dilution 1:5,000; cat. no. BM2004; Wuhan Boster Biological Technology Co., Ltd.) and detected using enhanced chemiluminescence (ECL) substrate (Cell Signaling Technology, Inc.). Appropriate positive and negative controls were run simultaneously. Western blot analysis was repeated 2-4 times. The protein bands were analyzed and quantified using a Bio-Profil Image Analysis system (Vilber Lourmat, Marne La Vallee, France), and the protein expression levels were expressed in relative units (RU). The results were normalized to actin.

Statistical analysis. The statistical software package SPSS 21.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. The experimental results are presented as the mean \pm standard deviation (mean \pm SD). The data

Table I. Immunohistochemical staining of LKB1 in CRC and normal colon mucosa.

Groups	Total no. N	Strong positive staining n (%)	Positive staining n (%)	Negative staining n (%)
Normal tissue	10	10 (100.0)	0 (0)	0 (0)
Inflammatory polyp	21	0 (0)	12 (57.1)	9 (42.9)
Adenocarcinoma/mucinous adenocarcinoma	65	0 (0)	28 (43.1)	37 (56.9)

LKB1, liver kinase B1 gene; CRC, colorectal cancer.

Table II. Association between clinicopathological factors and LKB1 protein expression as determined by IHC.

Parameter	Cases	LKB1		χ^2	P-value
		Positive	Negative		
Sex					
Male	35	19	16	0.215	0.643
Female	30	18	12		
Age (years)					
≤ 60	36	22	14	0.041	0.839
> 60	29	17	12		
Degree of differentiation					
I	20	15	5	2.550	0.279
II	31	18	13		
III	14	7	7		
Infiltrating depth					
T1+T2	35	17	18	4.205	0.004
T3	30	22	8		
Lymph node metastasis					
No	37	15	22	9.802	0.002
Yes	28	22	6		

LKB1, liver kinase B1 gene; IHC, immunohistochemistry.

analysis was performed with one-way ANOVA and χ^2 tests. One-way analysis of variance with least significant difference (LSD) post hoc test for multiple comparisons was performed. P-value < 0.05 was considered to indicate a statistically significant result.

Results

Expression of LKB1 protein in CRC and normal colon mucosa. LKB1 protein expression was evaluated using IHC staining in 96 cases. LKB1 protein expression and staining scores were observed in the cytoplasm and the nucleus. The expression of LKB1 was detected in the cytoplasm and nuclei of normal colon tissue, and positive expression was observed in 10 cases (100%). Positive expression of LKB1 was identified in 12 (57.1%) of the inflammatory polyp tissue samples examined, and negative expression was identified in 9 cases (42.9%). LKB1 expression was detected in the cytoplasm and nucleus of the CRC cells, and positive expression was observed in 28 cases (43.1%), while negative expression was detected

in 37 cases (56.9%). LKB1 expression in different tissues was significantly different ($P < 0.01$; Table I and Fig. 1).

The association between LKB1 expression and clinicopathological factors was analyzed in patients with CRC (Table II). The positive expression of LKB1 was significantly associated with the depth of invasion and lymph node metastasis ($\chi^2 = 4.205$ and 9.802 ; $P < 0.01$). However, LKB1 expression was not associated with sex, age or the degree of differentiation ($\chi^2 = 0.215$, 0.041 and 2.550 ; $P > 0.05$).

Effect of LKB1 siRNA on the morphology of LoVo cells. After transfection, the morphological changes in the different groups were observed by inverted microscope. There was no significant difference between the negative control group and the blank control group. The apoptotic cells in the present study refer to cells that can be seen to float in the culture solution. Compared with the negative control group, cell proliferation was increased and the number of cells was increased in the LKB1 siRNA group, but there was a small number of apoptotic cells, indicating toxicity to some cells (Fig. 2).

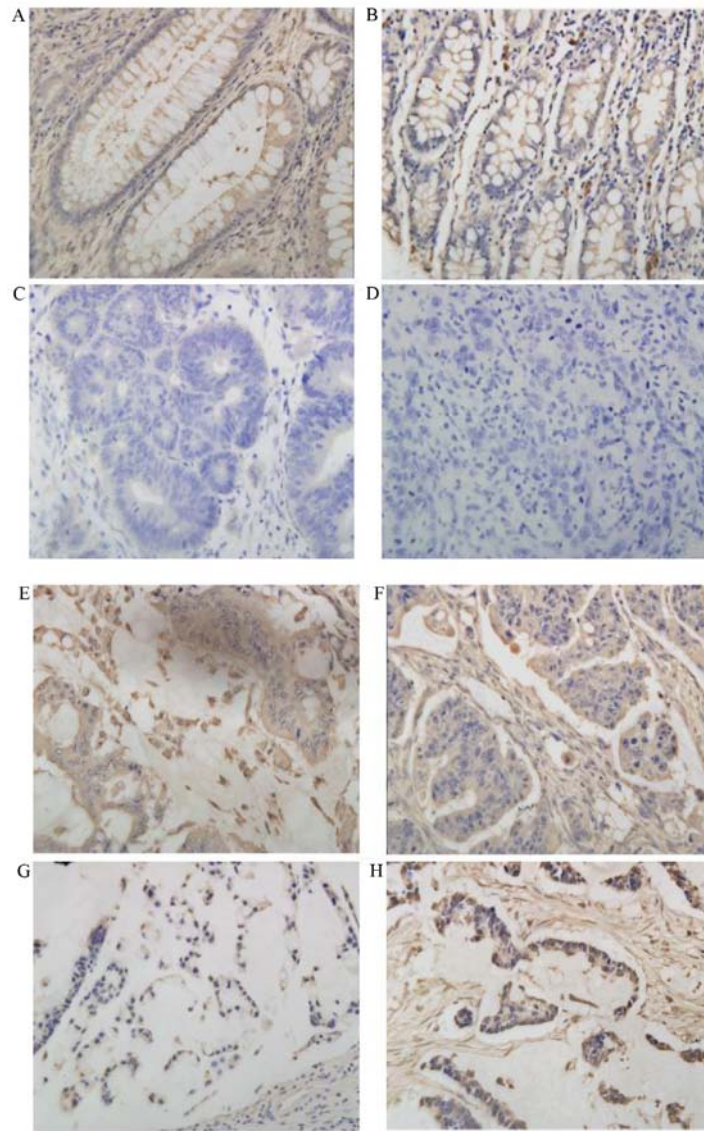


Figure 1. Immunohistochemical staining of LKB1 in colorectal carcinoma tissue sections (magnification, x200). (A and B) Normal tissue (positive). (C and D) Adenocarcinoma (negative). (E and F) Adenocarcinoma (positive). (G and H) Adenocarcinoma: Mucinous adenocarcinoma (positive). LKB1, liver kinase B1 gene.

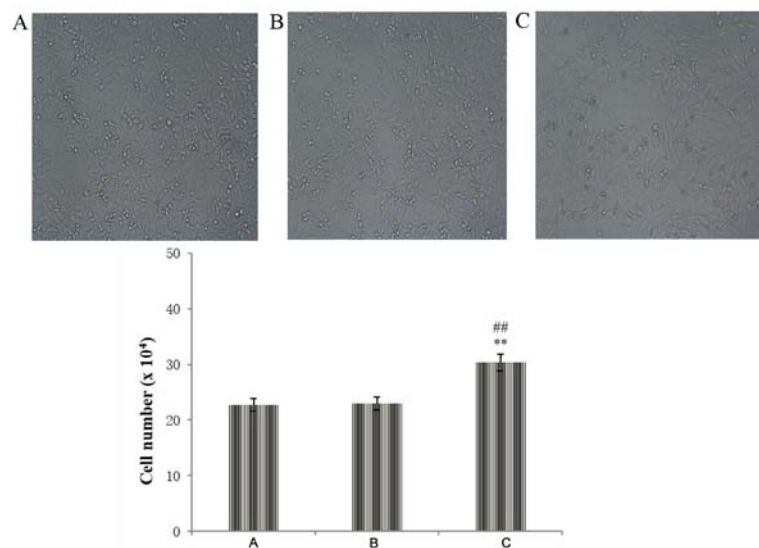


Figure 2. Effect of LKB1 siRNA on the morphological changes of LoVo cells magnification, x200). (A) Blank control group. (B) Negative control group. (C) LKB1 siRNA group. **P<0.01 vs. blank control group; ##P<0.01 vs. negative control group. LKB1, liver kinase B1 gene.

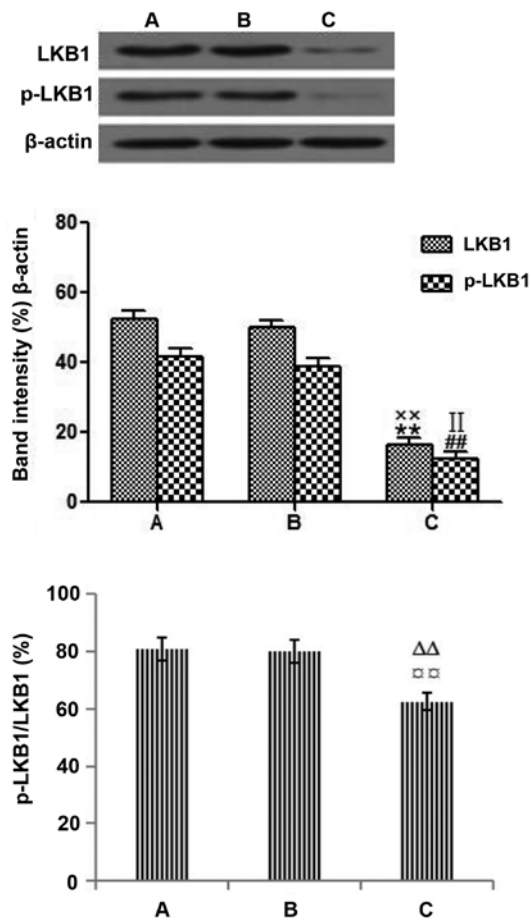


Figure 3. Western blot detection of LKB1 protein expression. (A) Blank control group. (B) Negative control group. (C) LKB1 siRNA group. ^{**}P<0.01 (LKB1), ^{##}P<0.01 (p-LKB1) vs. blank control group; ^{xx}P<0.01 (LKB1), ^{##}P<0.01 (p-LKB1) vs. negative control group. ^{ΔΔ}P<0.01 (p-LKB1/LKB1) vs. blank control group; ^{ΔΔ}P<0.01 (p-LKB1/LKB1) vs. negative control group. LKB1, liver kinase B1 gene; p, phosphorylated.

Western blotting detection of transfected cells. Following transfection of the LoVo cells with LKB1 siRNA for 48 h, western blotting was performed. LKB1 siRNA was synthesized by Guangzhou RiboBio Co., Ltd. The company tested that siRNA 001 had the best transfection efficiency. According to the company's synthetic results, the present study used siRNA-001 (data not shown). The expression levels of LKB1 and p-LKB1 in the LKB1 siRNA group were markedly decreased compared with those in the blank control group. Results indicate that p-LKB1/LKB1 expression levels were lower in the LKB1 siRNA group, compare to the negative control group (Fig. 3). The results confirmed that LKB1 siRNA had been successfully transfected into the LoVo cells.

Effect of metformin on cell proliferation as detected by CCK-8 assay. Metformin significantly inhibited the proliferation of LoVo cells in a dose- and time-dependent manner. The IC₅₀ of metformin was 20 mmol/l after 48 h of treatment (Fig. 4, Table III).

Effect of metformin on the morphology of LoVo cells. Compared with the blank control group, treatment with metformin resulted in apoptotic morphological changes in LoVo cells.

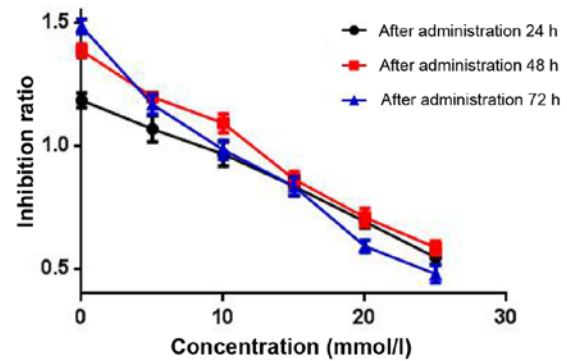


Figure 4. Effect of metformin on LoVo cell proliferation at 24, 48 and 72 h. Data are presented as the mean ± SD (n=6).

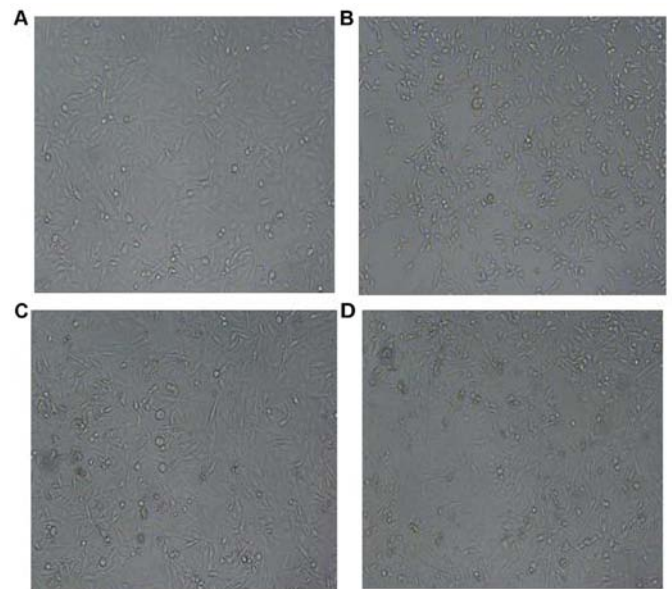


Figure 5. Effect of metformin on the morphology of LoVo cells (magnification, x200). (A) Blank control group. (B) Metformin group. (C) LKB1 siRNA group. (D) LKB1 siRNA+metformin group. LKB1, liver kinase B1 gene.

Under the light microscope, we observed that the shape of apoptotic cells became smaller, rounded and the adherent cells shrank and desquamated. The LKB1 siRNA group indicated that deletion of LKB1 led to cell proliferation (Fig. 5).

Effects of LKB1 on the migration and invasion of LoVo cells. A Transwell chamber assay was used to determine the migration and invasion abilities of LoVo cells. As shown in Figs. 6 and 7, the migration and invasion capabilities were clearly increased in the LKB1 siRNA group compared with the blank control group (P<0.01). The results demonstrated that the deletion of LKB1 increased the migration and invasion capacities of CRC cells. The results of the Transwell chamber experiments in the study indicated that the migration and invasion abilities of the CRC cells with deletion of LKB1 were enhanced, which indicated that the metastasis of CRC was enhanced.

To further explore whether the LKB1 signaling pathway is associated with the migration and invasion of LoVo cells, LoVo cells were treated with 20 mmol/l metformin for 48 h. As shown in Figs. 6 and 7, the migration and invasion

Table III. Inhibitory rate of cell growth of LoVo cells treated with metformin by CCK-8 assay.

Group	24 h	48 h	72 h
Blank control group	0.000+0.000	0.00+0.00	0.00+0.00
Negative control group	1.186+0.029	1.388+0.027	1.486+0.029
Metformin group (mmol/l)			
5	1.070+0.052 ^a	1.199+0.099 ^{a,b}	1.169+0.040 ^{a,b}
10	0.967+0.049 ^{a,c}	1.093+0.038 ^{a-c}	0.984+0.038 ^{a-c}
15	0.084+0.038 ^{a,c}	0.086+0.031 ^{a-c}	0.084+0.038 ^{a-c}
20	0.069+0.025 ^{a,c}	0.513+0.037 ^{a-c}	0.710+0.025 ^{a-c}
25	0.547+0.027 ^{a,c}	0.587+0.028 ^{a-c}	0.480+0.035 ^{a-c}

Metformin group vs. negative control group (^aP<0.05). Difference in terms of time was statistically significant in the same concentration group (^bP<0.05). Difference in terms of concentration was statistically significant in the same time group (^cP<0.05).

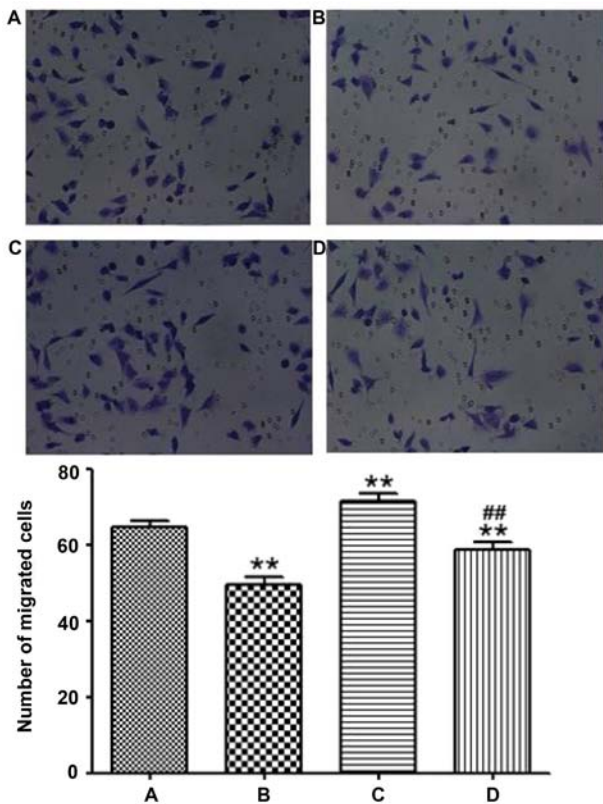


Figure 6. Migration of CRC cells in the different groups (magnification, x200). (A) Blank control group. (B) Metformin group. (C) LKB1 siRNA group. (D) LKB1 siRNA+metformin group. ^{**}P<0.01 vs. blank control group, ^{##}P<0.01 (p-LKB1) vs. LKB1 siRNA group. LKB1, liver kinase B1 gene; p, phosphorylated; CRC, colorectal cancer.

capacities were decreased in the metformin group compared with the blank control group. The migration and invasion capacities were decreased in the LKB1 siRNA+metformin group compared with the LKB1 siRNA group. The results of the present study suggest that metformin may be associated with the inhibition of migration and invasion-associated protein expression in LoVo cells.

Effects of metformin on the expression of LKB1 and p-LKB1 in LoVo cells. To confirm the effect of metformin on the

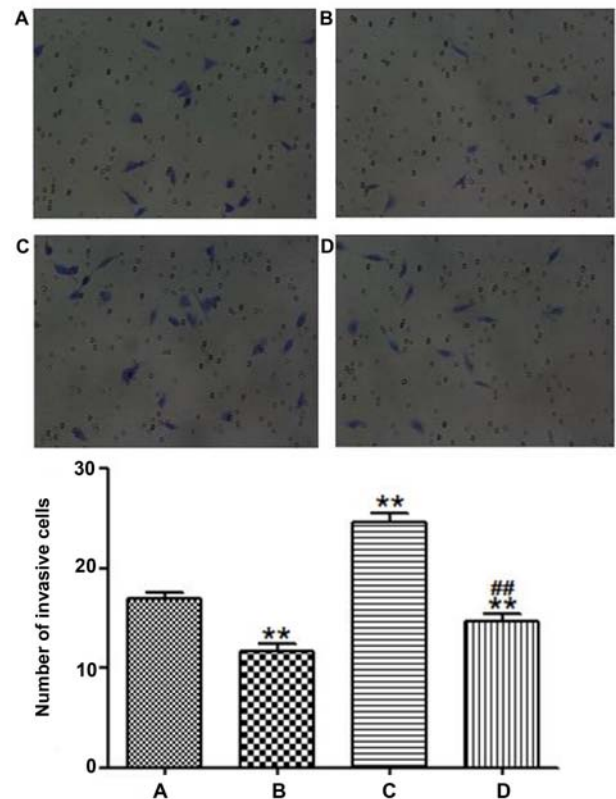


Figure 7. Invasion of CRC cells in the different groups (magnification, x200). (A) Blank control group. (B) Metformin group. (C) LKB1 siRNA group. (D) LKB1 siRNA+metformin group. ^{**}P<0.01 vs. blank control group, ^{##}P<0.01 (p-LKB1) vs. LKB1 siRNA group. LKB1, liver kinase B1 gene; p, phosphorylated; CRC, colorectal cancer.

expression of LKB1 and p-LKB1 in LoVo cells, western blot analysis was performed. The protein expression levels of LKB1, p-LKB1 and p-LKB1/LKB1 were significantly reduced in the LKB1 siRNA+metformin group compared with the blank control group (P<0.01). The protein expression levels of LKB1, p-LKB1 and p-LKB1/LKB1 were significantly increased in the LKB1 siRNA+metformin group compared with the LKB1 siRNA group (P<0.01; Fig. 8).

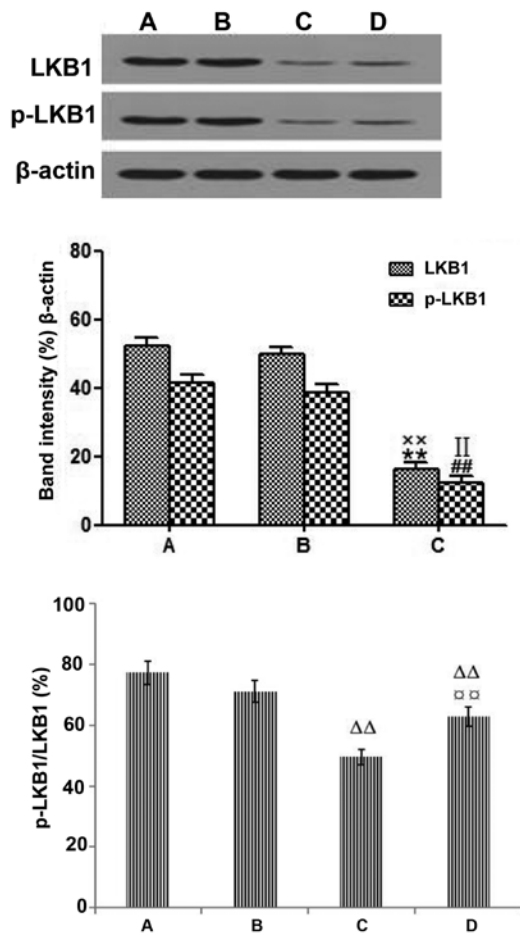


Figure 8. Effects of metformin on the expression of LKB1 and p-LKB1 in CRC LoVo cells. Western blot analysis showed the expression levels of LKB1 and p-LKB1 in LoVo cells. (A) Blank control group. (B) Metformin group. (C) LKB1 siRNA group. (D) LKB1 siRNA+metformin group. ^{**} $P < 0.01$ (LKB1) vs. blank control group, ^{##} $P < 0.01$ (p-LKB1) vs. blank control group; ^{xx} $P < 0.01$ (LKB1) vs. LKB1 siRNA group, ^{##} $P < 0.01$ (p-LKB1) vs. LKB1 siRNA group. ^{ΔΔ} $P < 0.01$ (p-LKB1/LKB1) vs. blank control group; ^{∞∞} $P < 0.01$ (p-LKB1/LKB1) vs. LKB1 siRNA group. LKB1, liver kinase B1 gene; p, phosphorylated; CRC, colorectal cancer.

Effects of metformin on the expression of AMPK and p-AMPK in LoVo cells. Western blot analysis showed that the expression of AMPK was not significantly different among the 4 groups ($P > 0.05$). The expression level of p-AMPK was significantly increased in the metformin group compared with the blank control group ($P < 0.01$). The expression of p-AMPK and p-AMPK/AMPK were significantly decreased in the LKB1 siRNA group compared with the blank control group ($P < 0.01$). However, the expression level of p-AMPK and p-AMPK/AMPK in the LKB1 siRNA+metformin group were much higher than that in the blank control group, suggesting that the expression of p-AMPK may play a role in CRC. Treatment with metformin increased the protein expression of p-AMPK compared with that of the LKB1 siRNA group, but it did not increase the expression of AMPK in the LKB1 siRNA+metformin group, which indicated that the effect of metformin on the p-AMPK protein expression was dependent on LKB1 ($P < 0.01$; Fig. 9). These results suggest that further investigation of the biological functions of the activation of AMPK in CRC is needed.

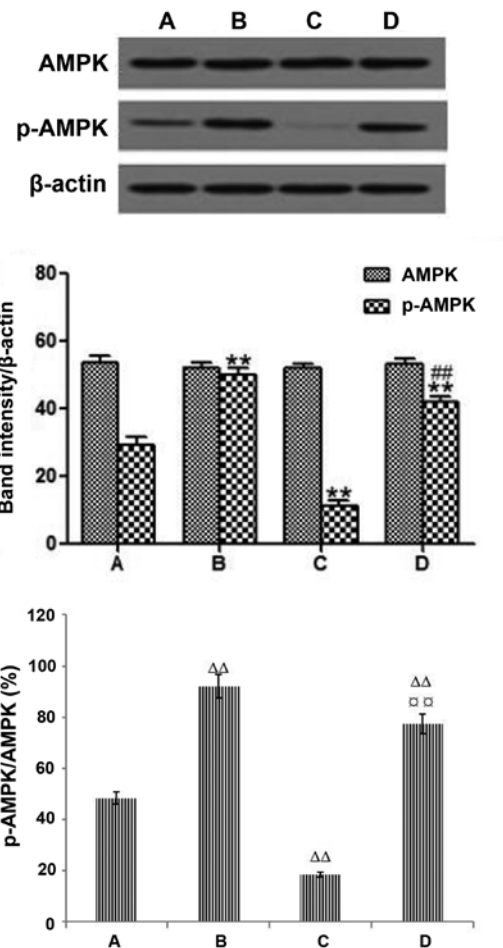


Figure 9. Effects of metformin on the expression of AMPK and p-AMPK in CRC LoVo cells. Western blot analysis showed the expression levels of AMPK and p-AMPK in LoVo cells. (A) Blank control group. (B) Metformin group. (C) LKB1 siRNA group. (D) LKB1 siRNA+metformin group. ^{**} $P < 0.01$ (AMPK) vs. blank control group, ^{##} $P < 0.01$ (p-AMPK) vs. LKB1 siRNA group. AMPK, AMP-activated protein kinase. ^{ΔΔ} $P < 0.01$ (p-AMPK/AMPK) vs. blank control group; ^{∞∞} $P < 0.01$ (p-AMPK/AMPK) vs. LKB1 siRNA group. LKB1, liver kinase B1 gene; AMPK, AMP-activated protein kinase; p, phosphorylated; CRC, colorectal cancer.

Effects of metformin on the expression of AKT and p-AKT in LoVo cells. Western blot analysis showed that the expression of AKT was not significantly different among the 4 groups ($P > 0.05$). Treatment with metformin decreased the protein expression of p-AKT compared with that of the blank control group. The LKB1 siRNA group had increased protein expression of p-AKT. The protein expression of p-AKT and p-AKT/AKT were reduced in the LKB1 siRNA+metformin group compared with the LKB1 siRNA group. These results indicated that metformin has an effect on p-AKT protein expression and suggested that the AKT signaling pathway may be associated with the metformin-induced changes in the expression of migration and invasion-associated proteins in LoVo cells (Fig. 10).

Discussion

LKB1 gene expression is closely related to the development and metastasis of colorectal cancer (CRC). By detecting the expression of LKB1 in CRC, we can provide a reference index

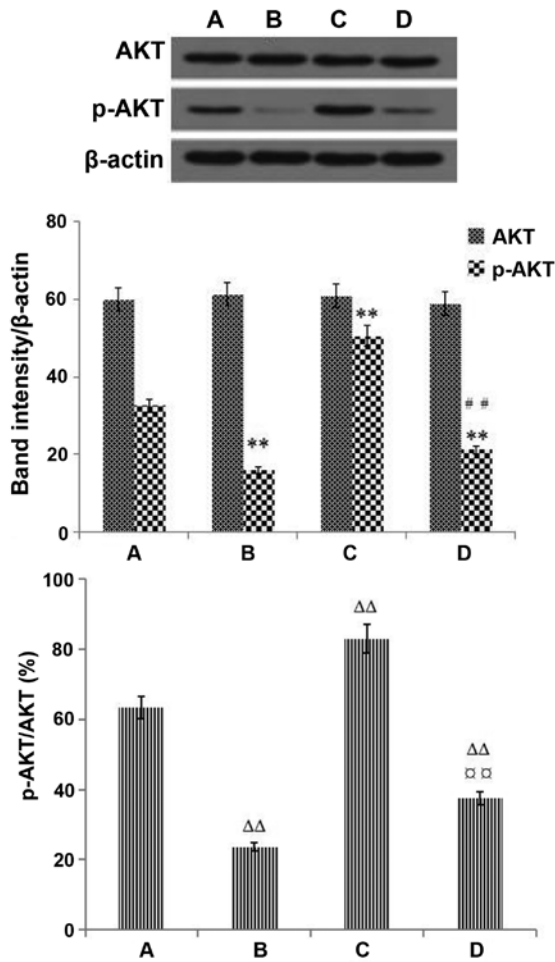


Figure 10. Effects of metformin on the expression of AKT and p-AKT in CRC LoVo cells. Western blot analysis showed the expression levels of AKT and p-AKT in LoVo cells. (A) Blank control group. (B) Metformin group. (C) LKB1 siRNA group. (D) LKB1 siRNA+metformin group. **P<0.01 (AKT) vs. blank control group, ##P<0.01 (p-AKT) vs. LKB1 siRNA group. ^{△△}P<0.01 (p-AKT/AKT) vs. blank control group; ^{°°}P<0.01 (p-AKT/AKT) vs. LKB1 siRNA group. LKB1, liver kinase B1 gene; AKT, protein kinase B; p, phosphorylated; CRC, colorectal cancer.

for diagnosing the depth of invasion and lymph node metastasis. It has been reported that the mutation rate of LKB1 in sporadic left colon cancer is 52.6% (26). In the present study, IHC results showed that expression of LKB1 as a tumor-suppressor gene was strongly positive in normal colorectal tissue, indicating that it can inhibit the occurrence of CRC. The expression of LKB1 was reduced in inflammatory polyp tissue. However, the expression of LKB1 was significantly decreased in adenocarcinoma and mucinous adenocarcinoma tissues, inducing the invasion and metastasis of CRC. These results provide a reference for further investigation of the role of LKB1 in the invasion and metastasis of CRC cells from the perspective of cytology.

New methods based on siRNA can effectively reduce tumor cell volume and inhibit tumor cell repair. siRNA technology has brought new possibilities for gene therapy for CRC patients (13). In the present study, LKB1 siRNA was transfected into LoVo cells to observe the effect of LKB1 on the invasion and metastasis of CRC and the potential underlying mechanism of that effect. As an important

tumor-suppressor gene, LKB1 plays a key regulatory role in the LKB1/AMPK/AKT signaling pathway. The results of the western blot analysis showed that the deletion of LKB1 led to a decrease in p-AMPK in the downstream pathway, which promoted p-AKT, tumor cell proliferation and angiogenesis and enhanced the migration and invasion of CRC cells. The present study used CRC cells to observe the effect of LKB1 on invasion and metastasis of CRC cells. This *in vitro* experiment had limitations. Further research will be carried out by us in *in vivo* experiments to observe the effect of LKB1 on CRC metastasis in nude mice.

Studies have shown that metformin-induced AMPK activation can reduce protein synthesis and cell proliferation in CRC (27,28). Metformin induces cell cycle arrest or apoptosis, activates the immune system, improves immunity and eliminates cancer stem cells. Increasing numbers of clinical studies have shown that metformin as a typical adjuvant chemotherapy can reduce the risk of invasion and metastasis in cancer patients, resulting in more successful chemotherapy (29). Therefore, we used metformin treatment to observe the effect of metformin on the motility and invasiveness of CRC cells to investigate the possible mechanism by which LKB1 acts on the AMPK-AKT signaling pathway in CRC metastasis. Metformin-induced AMPK activation reduced protein synthesis and cell proliferation and inhibited p-AKT production in CRC cells, thereby inhibiting tumor metastasis, promoting tumor cell apoptosis and decreasing tumor cell migration and invasion. Interestingly, our experiments showed that metformin did not directly activate the phosphorylation of LKB1 (p-LKB1). Metformin inhibited the invasion and metastasis of CRC by activating p-AMPK, thereby inhibiting the activation of p-AKT.

In conclusion, our findings demonstrates that LKB1 plays an important role in the invasion and metastasis of CRC by activating AMPK, negatively regulating the AKT signaling pathway and regulating gene expression, which may be a new anti-angiogenesis and antimetastasis target in CRC treatment. Mutation or deletion of LKB1 repair is expected to be a new therapeutic target or clinical biomarker for the treatment of the invasion and metastasis of CRC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

YZ designed the experiment, analyzed and explained the data, modified the manuscript, and approved the final version of the manuscript. YC performed the cell experiments and data analysis and interpretation and drafted the manuscripts. YL conceived the experiments, obtained, compiled and analyzed experimental data, participated in the drafting of the manuscripts and approved the final manuscript. HY performed the immunohistochemistry experiments and carried out the data analysis. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Hubei University of Science and Technology (Xianning, China). The committee's reference no. is 201601001. The colon cancer tissue microarray was purchased from Fanpu Biotech, Inc. The company ensured ethical approval from the patients, and patient consent for publication; Clinical Research Protocol (version no. 2016LC001) and informed consent (version no. 2016TYS001).

Patient consent to publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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