Original Article

Epigenetic silence of lumican inhibits the motility of colon cancer via inactivating MAPK signaling in vitro and in vivo

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Received March 9, 2017; Accepted April 13, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: Objective: To explore the effects of lumican on invasion and migration abilities of colon cancer cells and the relevant mechanism. Methods: The expression of lumican in four colon cancer cell lines (SW480, SW620, HCT-8 and LOVO) and the human colonic epithelial cells CCD-18Co was detected by western blotting. Lumican was silenced by constructing lentivirus and transfecting into SW480 and HCT-8 cells. The efficiency of silencing was detected by qRT-PCR and western blotting. The effect of lumican siRNA on invasion and migration of colon cancer cells was detected by transwell assay and wound scratch assay. The expression of metastasis-related proteins vascular endothelial growth factor (VEGF) and metal matrix proteinase (MMP)-9 was detected by western blotting. The level of ERK1, p-ERK1, JNK and p-JNK was also detected by western blotting. Finally, the effects of lumican on tumor growth and metastasis were detected in colon cancer xenograft. Results: The expression level of lumican was significantly increased in colon cell lines compared with CCD-18Co. Lumican was then silenced by transfecting siRNA into SW480 and HCT-8 cells. The decreased invasive cell number with reduced wound closing rate was detected in SW480 and HCT-8 cells transfected with lumican siRNA compared with control group. The decreased level of metastasis-related proteins VEGF and MMP-9 further convinced the inhibitory effect of lumican siRNA transfection on the motility of colon cancer. In addition, the expression of ERK/JNK pathway proteins was strongly suppressed under lumican siRNA treatment. Finally, the in vivo experiment revealed that lumican-siRNA strongly reduced the tumor growth and tumor volume. Besides, lumican-siRNA transfection inhibited tumor metastasis via suppressing the expression of metastasis-related proteins. The level of p-ERK1/2 and p-JNK was also reduced treated with lumican-siRNA. Conclusions: The inhibiting of lumican restrains the invasion and migration abilities of colon cancer and may through inhibiting ERK/JNK signaling pathway. Lumican may become a biological marker for predicting progression and prognosis of colon cancer.

Keywords: Lumican, colon cancer, invasion, migration, ERK

Introduction

Colon cancer is one of the most malignant tumors in China, with increasing incidence and mortality. According to relevant statistical data, the mortality of colon cancer has ranked the second among all cancer-related deaths, and half of the patients have distant metastasis [1, 2]. The onset of colon cancer is occult, without evident symptoms in the early stage. More severe, colon cancer in the middle and advanced stages was often accompanied by tumor metastasis. With rapid advances in the surgery-based comprehensive treatment, the prognosis of patients with colon cancer has been improved significantly, but the 5-year survival rate is still around 50% [3]. Therefore, investigation on mechanisms of invasion and migration and searching for the targets for early diagnosis and monitoring on the treatment effect are constantly the focus in studies on colon cancer.

The extracellular matrix (ECM) is closely related to tumor invasion and migration. Pathological changes can be observed during tumor infiltration and metastasis, including adhesion of the tumor cells to ECM and invasion into the matrix [4-6]. Lumican, a poly-membrane protein, is an essential component of ECM. The human Lumican gene is located at chromosome 12q21.3-
Lumican inhibits colon cancer cells via MAPK signaling pathway

q22 [7]. Lumican plays an important role in regulating the balance of extracellular water. Lumican has also been suggested to regulate cell proliferation and metastasis in various tumors [8]. There is a relationship between lumican expression and growth of tumor cells in the interstitial tissues adjacent to cells; Lumican expression is possibly an important factor in invasion of pancreatic tumor cells [9]. Beyrau et al. has shown that lumican is involved in the transmission of tumor inflammation-related signals by binding to β2, αM and αL integrin subunits of polymorphonuclear leukocytes. Besides, inflammation response may destroy the body’s immune system, leading to the occurrence of tumors [10]. However, the effect of lumican in colon cancer has been seldom reported, especially the effect of lumican on tumor invasion and migration and the related mechanisms.

In this study, we investigated the effect of lumican on the motility of the colon cancer and found that lumican was overexpressed in colon cell lines compared with CCD-18Co cells. The inhibiting of lumican effectively restrained the invasion and migration abilities of colon cancer in vitro and in vivo and may through inhibiting ERK/JNK signaling pathway.

Materials and methods

Cell lines and culture

The human colon cancer cell lines SW480, SW620, HCT-8, LOVO and the human colonic epithelial cells CCD-18Co were purchased from Wuhan University Cell Collection Center. Cell culture conditions: SW480, SW620 and CCD-18Co were cultured in RPMI 1640 containing 10% fetal calf serum at 37°C, 5% CO₂; HCT-8 and LOVO were cultured in RPMI DMEM containing 10% fetal calf serum at 37°C, 5% CO₂. The fetal calf serum was purchased from Gibco. The RPMI 1640 and RPMI DMEM media were obtained from HyClone.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from each cell line were extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The extracted RNA was then eluted with RNase-free water and stored at -80°C.

qRT-PCR was performed by using SYBR-green PCR Master Mix in a Fast Real-time PCR 7500 System (Applied Biosystems). The RT-PCR primers for lumican were purchased from GeneCo-poeia (SanDiego, California, USA). The specific primers were as follows: lumican (5’-TCGAGCATGCTCTCCTAT-3’ forward and 5’-TGGTCCCAGGTCTTACAGAA-3’ reverse), GAPDH was used as the internal control of the mRNA. Fold change of lumican was calculated by the equation $2^{ΔΔCt}$.

Western blot analysis

Cell samples were lysed in lysis buffer (Beyotime, China). The samples mixed with loading buffer were incubated in boiling water for 10 min. 20-30 μg protein was separated through SDS-PAGE and then transferred onto Polyvinylidene Fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were blocked in PBS with 0.1% Tween 20 containing 5% nonfat milk for 2 h at room temperature, and then were incubated with the primary antibodies and the corresponding HRP-conjugated secondary antibodies. The primary antibodies of lumican, vascular endothelial growth factor (VEGF), metal matrix proteinase (MMP)-9, ERK1, p-ERK1, JNK and p-JNK were purchased from Abcam (ab168348, ab17942, ab50011, ab179461 and ab76572). Membranes were extensively washed several times. Proteins were detected using a ChemiDoc XRS imaging system and Quantity One analysis software (Bio-Rad, San Francisco, California, USA). U6 and GAPDH (Abcam) were used as endogenous references.

Cell transfection

SiRNA fragments targeting lumican were designed and purchased from Invitrogen (USA). The scramble fragments were designed as the negative control of lumican. The SW480 and HCT-8 cells were seeded in 24-well plates at 1 × 10⁵ cells per well. lumican siRNA and siRNA scramble were transfected into SW480 and HCT-8 cells using Lipofectamine 2000 according to the protocol (Invitrogen, USA). Cells were harvested for subsequent experiments after transfection for 24 hours.

Wound-healing assay

Wound-healing assay was performed to evaluate the migration rate of SW480 and HCT-8
Lumican inhibits colon cancer cells via MAPK signaling pathway

Figure 1. Lumican is highly expressed in colorectal cancer cell lines. Relative expression of lumican in colon carcinoma cell lines (SW480, SW620, HCT-8 and LOVO) and normal human colonic epithelial cells lines (CCD-18Co) was detected through qRT-PCR (A) and western blot (B) (**P < 0.01 versus CCD-18Co). (C) Relative protein expression of lumican in each cells was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean ± SD of three experiments.

Figure 2. SiRNA transfection effectively reduces the level of lumican. Two colon cancer cell lines SW480 and HCT-8 were transfected with lumican siRNA or scramble, respectively. A. The expression of lumican in SW480 and HCT-8 cells was detected through western blotting. GAPDH was used as an endogenous reference. B. Histogram represents the statistical analysis of the changes in the expression of lumican (**P < 0.01 versus scramble group).

 Colon cancer xenografts

Specific pathogen-free (SPF) athymic nude mice (male, six to eight weeks of age) were housed and manipulated according to the protocols approved by the Experimental Animal Center of the Southwest Medical University. For researching tumorigenicity of lumican in vivo, SW480 cells were transfected with lumican siRNA or siRNA scramble, respectively. Each mouse was subcutaneously inoculated with 1 × 10⁶ SW480 cells transfected with lumican siRNA or scramble (fluorescent-labeled) in 50% Matrigel (BD Biosciences). After the development of a palpable tumor, the tumor volume was monitored every 6 days and assessed by measuring the 2 perpendicular dimensions using a caliper and the formula (a × b²)/2, where a is the larger and b is the smaller dimension of the tumor. At 30 days after inoculation, the mice were killed and tumor weights were assessed. Tumors from each mouse were randomly selected for immunohistochemical (IHC) analysis. All the animal experiments were performed according to relevant national and international guidelines and were approved by the Animal Experimental Ethical Committee.
Lumican inhibits colon cancer cells via MAPK signaling pathway

Statistical analysis

The results are presented as the mean ± the standard error of the mean of 3 replicates. Differences between means were analyzed using Student’s t test. The difference was considered statistically significant at \( P < 0.05 \).

Results

**Lumican is highly expressed in colorectal cancer cell lines**

To determine whether lumican was involved in the development of colon cancer, the expression of lumican in colorectal cancer tissues and cell lines was detected through qRT-PCR. As shown in Figure 1A, the RNA expression level of lumican was significantly increased (range from 7-fold to 13-fold) in a panel of human colon cancer cell lines compared with normal human colonic epithelial cells lines CCD-18Co (\(^*\*P < 0.01\), Figure 1A). The western blot analysis further convinced the elevated expression of lumican in colon cancer cells compared with CCD-18Co group (Figure 1B and 1C). These results suggest that lumican is highly expressed in colon cancer.

**SiRNA transfection effectively reduces the level of lumican**

Considering the high expression of lumican in colon cancer cells, we then silenced the expression of lumican by transfecting siRNA fragments specific for targeting lumican into SW480 and HCT-8 cells. The results indicated that the mRNA and protein expression level of lumican was significantly decreased in both SW480 and HCT-8 cells (\(^*\*P < 0.01\), Figure 2).

**The inhibition of lumican reduces cell motility of colorectal cancer**

We next investigated the role of lumican siRNA in regulating the migration and invasion ability in colon cancer cells. The number of invasive cells was decreased over 7 times in SW480 and HCT-8 cells transfected with lumican siRNA compared with scramble group (\(^***P < 0.001\), Figure 3). Besides that, the results of wound healing assays showed a large closure of the gap in scramble group, whereas, a negligible effect on the closing rate of scratch wounds was seen in the lumican siRNA group compared with scramble group (\(^***P < 0.01\), Figure 4). Moreover, the expression level of tumor metastasis-related proteins (MMP-9 and VEGF) were largely decreased by lumican siRNA (\(^***P < 0.01\), Figure 5). These results indicate that the inhibition of lumican reduces cell motility of colorectal cancer.

**The inhibition of lumican inactivates ERK/JNK signaling**

The related signaling was then investigated in colon cancer cells. No significant change was

![Figure 3. The inhibition of lumican reduces cell invasion ability of colorectal cancer. A. The invasive cells were detected by transwell invasion assays in SW480 and HCT-8 cells. B. Histogram represents the statistical analysis of Wound-healing assay. Data are represented as the mean ± SD of three experiments. **P < 0.001 versus scramble group.](image)
Lumican inhibits colon cancer cells via MAPK signaling pathway

observed in the expression of ERK1 and JNK (\(P > 0.05\)). However, the level of p-ERK1 and p-JNK was obviously down-regulated in SW480 and HCT-8 cells transfected with lumican siRNA compared with scramble group (\(^{**}P < 0.01\), Figure 6). The results above indicate that the inhibition of lumican inactivated ERK/JNK signaling in colon cancer cells.

The inhibition of lumican suppresses tumor growth and metastasis in colon cancer xenografts

To investigate the effect of lumican siRNA on colon cancer growth and metastasis \(in\) v\(ivo\), xenograft mouse model was created by subcutaneous injection of SW480 cells pretreated with lumican siRNA or scramble to SPF nude mice. Lumican siRNA effectively suppressed tumor formation and tumor volume compared with scramble group (\(^{***}P < 0.001\), Figure 7A, 7B). The level of tumor metastasis-related proteins MMP-9 was significantly decreased in lumican siRNA treated mice tissue compared with scramble group (Figure 7C). Besides that, the level of lumican, p-ERK1 and p-JNK was strongly decreased in lumican siRNA group mice compared with scramble group (\(^{**}P < 0.01\), Figure 7D, 7E). These results indicated that the lumican siRNA restrained the growth and metastases of colon cancer \(in\) v\(ivo\) and may inactivate the ERK1/JNK signaling pathway.

Discussion

Tumor infiltration and metastasis is a complicated process with multiple steps. To achieve metastasis, tumor cells must first infiltrate the ECM. Then, tumor cells can bind to ECM components, move to the infiltration area, pass through the matrix and result in metastasis \([11-14]\). Thus, ECM plays an essential role in tumor metastasis. Lumican is an important component of ECM. Many studies have shown that the leucine-rich proteoglycan family plays a major role in regulating cellular activities, including cell movement, tissue repair and tumor proliferation, as well as in ECM tissue hydration and collagen formation, etc \([15, 16]\). In recent years, several studies have demonstrated that lumican plays an critical part in tumor proliferation, apoptosis, infiltration and metastasis \([17-20]\).
Lumican inhibits colon cancer cells via MAPK signaling pathway

Our study investigated the effect of lumican on the motility of colon cancer. As a member of a small leucine-rich proteoglycan family, the overexpression of lumican has been reported in breast [20], colorectal [21], uterine cervical [22] and pancreatic cancers [9]. Seya T et al. indicated that lumican expression was a potential prognostic factor in patients with advanced colorectal cancer with nodal metastasis [21]. Reports also have shown that lumican overexpression is associated with poor prognosis of pancreatic cancer [9]. In accordance with these reports, high expression of lumican was detected in colon cancer cell lines compared with human colonic epithelial cells CCD-18Co, suggesting the tumorigenesis-promoting role of lumican.

The expression of lumican also correlates with the metastasis of various malignancies. Report indicated that lumican in stromal tissues plays an important role in the invasion of pancreatic cancer [9]. Report indicated that the increasing invasion of colorectal cancer with higher positive rate of lumican was observed in colon cancer tissues compared with healthy tissues [23], suggesting that lumican is closely related to the

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**Figure 5.** The inhibition of lumican suppresses the expression of metastasis proteins. (A) The expression of MMP-9 and VEGF in SW480 and HCT-8 cells were detected through Western blotting. GAPDH was used as an endogenous reference. Relative protein expression of MMP-9 (B) and VEGF (C) in SW480 and HCT-8 cells was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean ± SD of three experiments. **P < 0.01 versus scramble group.

**Figure 6.** The inhibition of lumican inactivates ERK/JNK signaling. A. The expression of ERK1, p-ERK1, JNK and p-JNK in SW480 and HCT-8 cells was detected through Western blotting. GAPDH was used as an endogenous reference. B. Relative protein expression of each protein in SW480 and HCT-8 cells was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean ± SD of three experiments. **P < 0.01 versus scramble group.
Lumican inhibits colon cancer cells via MAPK signaling pathway

Figure 7. The inhibition of lumican suppresses tumor growth and metastasis in colon cancer xenografts. SW480 cells pretreated with lumican siRNA or scramble were injected into BALB/c nude mice. A. Representative tumors from two groups were shown (n = 5). B. Tumor growth trend in SW480-lumican siRNA mice and SW480-scramble mice was showed. C. The production of migration markers MMP-9 in formalin-fixed, paraffin-embedded tumors from SW480-lumican siRNA mice and SW480-scramble mice was detected through IHC analysis. D. The expression of lumican, p-ERK1 and p-JNK in each group was detected through Western blotting. GAPDH was used as an endogenous reference. E. Relative protein expression of each protein was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean ± SD of three experiments. *P < 0.05, **P < 0.01 versus scramble group.

Metastasis of colorectal cancer. Consistent with these results, the wound closing rate and invasive cell numbers was decreased with restrained expression of VEGF and MMP-9 were measured in SW480 and HCT-8 cells transfected with lumican siRNA compared with scramble group. These results indicate that the inhibition of lumican effectively suppresses the motility of colon cancer.

MAPK (mitogen-activated protein kinases) is a class of intracellular serine/threonine protein kinase, playing an important role in cell proliferation, apoptosis and intercellular function synchronization [24]. The phosphorylation of MAPK induces the activation of some nuclear transcription factors and thereby regulating the biological behaviors and functions of cells [25]. Studies have suggested that the invasion and metastasis of breast cancer is associated with abnormal activation of ERK/MAPK signaling pathways [26]. In gastric cancer cells, protease-activated receptor-2 induces the expression of VEGF and cyclooxygenase-2 by activating the ERK1/2 and p38 signaling pathways, thereby promoting angiogenesis and metastasis of gastric cancer cells [27]. In this study, the expression levels of p-ERK1/2 and p-JNK was significantly decreased after silencing lumican, suggesting that reduced level of lumican down-regulated the expression of p-ERK1/2 and p-JNK, and lumican could influence the functions of colon cancer cells through inactivating the MAPK signaling pathway.

Taken together, our study investigated the role of lumican in the metastasis of colon cancer and found that lumican was overexpressed in colon cancer cell lines. After silencing lumican by siRNA, the invasion and migration abilities of colon cancer was significantly inhibited with reduced expression level of ERK1/JNK pathway. Finally, the in vivo experiment revealed that lumican siRNA strongly reduced the tumor growth and metastasis via suppressing the expression of metastasis-related proteins and ERK1/JNK pathway. These findings suggest that lumican may be involved in invasion and migration of colon cancer; and lumican may become a biological marker for predicting progression and prognosis of colon cancer.

Acknowledgements

This study was supported by Science and Technology Fund Project of Tianjin Municipal Health Bureau (Nos. 2012KY20).
Lumican inhibits colon cancer cells via MAPK signaling pathway

Disclosure of conflict of interest

None.

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Lumican inhibits colon cancer cells via MAPK signaling pathway


